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Active State Durations of Rat Gastrocnemius Muscle

By

B WILANDER

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Abstract

WILANDER B *Active state durations of rat gastrocnemius muscle* Acta physiol scand 1966 68 1-17

For rat gastrocnemius muscle it has been found that 1 The shortening velocity of an isotonically afterloaded contraction increases with the stimulation frequency up to a frequency of 40 imp/sec

stimulation with frequencies somewhat below that necessary for fusion it has been found that the mechanical latency is as short as after the first impulse (0.6 msec). The maximal rate of tension rise after an impulse is however considerably delayed and the implications of this finding are discussed in terms of the active state concept.

During an investigation of the properties of rat gastrocnemius muscle I found that the optimal stimulation frequency for an isotonic contraction against a small load was considerably higher than the lowest frequency giving maximal plateau tension during an isometric tetanus. A research program was then started to investigate the fundamental cause of this difference in response to stimulation frequency. Such a study is thought to be of some importance for research in muscle physiology as it is apparent that formerly the situation was often thought to be under experimental control when a fixed stimulation frequency was used throughout the contractions. During the course of this investigation a paper by Buller and Lewis appeared in which a similar difference in optimal stimulation frequency was described for rate of tension development and for peak tension during indirect stimulation of cat muscle (Buller and Lewis 1965).

According to Hill there exists for a period of time after an impulse a constant level of maximal intensity of the active state which is abruptly developed at the end of the latent period (Hill 1949 1953). In the present paper a distinction will be

drawn between an active state for shortening, which is indicated by the capacity of the muscle to reduce its length and an active state for tension maintenance, which is indicated by the capacity of the muscle to resist stretch, as suggested by Sandow (1960)

Sensitive determinations of the active state plateau duration have been made by superimposing contraction curves for one and two impulses on each other (Macpherson and Wilkie 1954). According to Ritchie, the duration of the active state plateau may also be determined from the time from the last impulse of a tetanic train to the beginning of relaxation after a correction for mechanical latency (Ritchie 1954 *b*). A method related to the latter is the determination of the fusion frequency (Mauriello and Sandow 1953, Ritchie 1954 *b*).

In several papers the duration of the active state plateau after a single impulse is reported to be of the same order of size as the duration of the active state plateau during a fully developed isometric tetanus (Ritchie 1954 *a*, Macpherson and Wilkie 1954, Bleckmann *et al.* 1963, Mårtensson and Skoglund 1964). The lastmentioned authors, however, express some doubt about the reliability of determinations of the duration of the active state plateau after a single impulse.

The present work will show that there is a considerable difference between a well marked active state plateau for shortening after the first impulse and what is generally considered as an active state plateau for tension maintenance when peak isometric tetanic tension has developed, the first active state plateau being very short. The validity of the active state concept will be discussed in relation to the present findings.

Methods

were covered with vaseline care being taken not to disturb the contractions with any extra damping. The femur was rigidly clamped in a horizontal position in a screw vice. One of the clamps passed

ed several times through the remaining soft tissues of the heel as a cathode and the bone clamp as a ground lead, the pulse duration being 0.25 or 0.30 msec.

Isometric contractions were recorded by means of a RCA 5734 mechano-electric transducer, the anode pin of which was mounted on the middle of a short brass bar fixed at both ends. The muscle was connected with this bar by means of a chain and a light calcaneus clamp. The bar was also provided with a light damping pot with a fixed vane. The static response of the system was found to be linear, even for very small tensions. The natural frequency of the system, with chain

shift and amplitude distortion due to resistances and stray capacitances of the electric system were found to be negligible, compared with those of the mechanical, the rise time for a step change being 0.03 msec. The stationary state phase lag of the system for periodic sinusoidal driving functions was calculated to be no more than 12 msec up to the natural undamped frequency, the apparent latency of a transient response being much less. The output of the isometric recording system was fed to a Tektronix 561 oscilloscope with a 72 dual trace amplifier, either directly or through high pass

12 g and the stiffness of the lever corresponded to 60 lb/cm at the muscle end.

For the recording of action potentials a Tektronix 122 preamplifier was used and the bipolar electrode consisted of 0.2 mm twisted copper wire, which was insulated except at the tips which were introduced into the distal end of the muscle.

Results

A comparison between the dependences upon stimulation frequency of isometric peak tension and isometric shortening velocity

The isometric contractions were recorded at the muscle length at which resting tension just disappears. At this length the tetanic tension soon reached a constant mean value, even at rather low stimulation frequencies. The tension was measured at 130 msec. of stimulation. For unfused tetani the tension was measured to the peak of the oscillations. Apparent fusion seemed to occur at about 200–225 imp/sec at the amplification used in the tension determinations. The isometric contractions were afterloaded and started at the optimum length for twitch peak tension against a load of 100 pond. A comparison on the active state plateau durations at different lengths is presented in the third section of the results. The shortening velocities were measured after about 10 msec of stimulation, when the velocity became fairly constant. At the lowest frequency slight oscillations sometimes occurred; the velocity was then measured over the peaks of these oscillations. The stimulation intensity was supramaximal, even for high frequency stimulation. For a discussion of the proper conditions for direct stimulation, see the next section. The recordings were made in an ascending or descending order of frequency. Every third contraction was a reference contraction at 180 imp/sec for isometric and 400 imp/sec for isometric contractions. Interpolated corrections were performed to compensate for slight

TABLE I A Isometric tetanic tension at 150 msec at different stimulation frequencies

Stimulation frequency imp/sec		130	160	180	200
Direct stimulation	Relative tension	96.1	98.2	100.0	100.9
	S.E.	0.61	0.55	0.31	0.26
	p	0.012	0.015	0.022	0.038
Indirect stimulation	Relative tension	96.3	99.0	100.0	101.1
	S.E.	0.57	0.17	0.27	0.23
	p	0.006	0.002	0.01	0.7

Tensions and velocities are presented in relation to the reference contractions at 180 and 400/sec the velocities for indirect stimulation being presented in relation to the velocity for direct stimulation at 400/sec. S.E. = standard error of the mean of the relative differences in animals between the adjacent stimulation frequencies. p = calculated risk of making a wrong decision when rejecting the

TABLE I B Isotonic shortening velocity at different stimulation frequencies

Stimulation frequency imp/sec		180	300	375
Direct stimulation	Relative velocity	79.8	90.7	96.8
	S.E.	1.26	0.17	1.36
	p	0.001	0.000	0.039
Indirect stimulation	Relative velocity	73.1	82.1	85.0

progressive changes in response. The largest total correction varied from 1% to 11% between the different preparations.

The dependence of isometric tension and of isotonic shortening velocity upon stimulation frequency were investigated both for direct and for indirect stimulation. The results are shown in Table I. Statistical calculations according to the common *t* test were performed on the relative differences of responses in animals between adjacent stimulation frequencies. Some calculations for larger frequency intervals are also shown. The deviates for the frequency intervals between 250 and 180 imp/sec, isometric registration were pooled and found to be approximately normally distributed, as small differences in similar animals often are. The tests were one tailed, as the possible direction of change is well known.

For isometric contraction the results are quite in agreement with the often-quoted conclusion of Cooper and Eccles (1930), that above the apparent fusion frequency no substantial increase in tension occurs. In this case the tension increases signifi-

225	250	275	300	400	
					300-225
101.4	101.5	101.5	101.9	99.1	
0.32	0.68	0.43	0.53		0.33
0.3	—	0.17	0.008		0.3
					275-200
101.2	101.3	101.7	101.3	99.8	
0.24	0.49	0.39	0.86		0.42
0.7	0.5	0.2	0.08		0.2

null hypothesis that no difference exists in favour of the hypothesis that a difference exists in an expected direction. In the last column to the right analyses of larger frequency intervals are presented. Number of animals: 1 A direct stimulation, data pooled from 7 rats, 5 differences for each frequency interval; 1 A indirect stimulation, 6 rats; 1 B, 5 rats on which records for indirect as well as direct stimulation were obtained.

400	425	450	500	
				425-375
100.0	102.7	100.6	101.6	
0.88	1.36	0.81		1.49
0.019	0.2	0.2		0.008
86.9	87.4	87.8	89.6	

cantly up to about 200 imp/sec for indirect and for direct stimulation and then seems to remain on a plateau within the limits of accuracy of a small number of experiments. At 400 imp/sec a decline due to some refractoriness is evident. In isotonic contractions during direct stimulation, however, the response increases significantly up to about 425 imp/sec, a frequency about twice as high as the corresponding frequency for isometric tension at 150 msec.

No statistical calculations are presented for the indirect isotonic data, the course of the velocity-frequency curves being rather irregular. The highest maximum is often not obtained until 500 imp/sec (cf. Buller and Lewis 1965). The velocities by indirect stimulation were always lower than those by direct stimulation and it is obvious that maximal shortening velocity is not attained by indirect stimulation. Such a difference was not evident for isometric contractions, maximal response being obtained at lower frequencies.

TABLE II A Results obtained from isometric contraction records for one and two impulses

Direct stimulation										Indirect stimulation	
Column 1	2	3	4					5	6	7	8
Animal number	Period of latency	L R start to tension minimum	L R start to separation Stimulation intervals					Shortest separation time	Stimulation interval for 5	Shortest separation time	Stimulation interval for 7
			08	09	10	11	12				
1	0.65	0.33	1.85	<i>1.44</i>	<i>1.42</i>	<i>1.44</i>	—	1.42	1.0	1.66	1.0
2	0.65	0.30	1.66	<i>1.36</i>	<i>1.36</i>	1.41	1.47	1.36	0.9 1.0	1.71	1.0
3	0.54	0.49	1.78	<i>1.59</i>	<i>1.55</i>	<i>1.66</i>	<i>1.71</i>	1.55	1.0	1.72	1.1
4	0.60	0.49	<i>1.37</i>	<i>1.36</i>	<i>1.39</i>	1.52	1.66	1.36	0.9	1.69	1.0
5	0.54	0.60	<i>1.58</i>	<i>1.58</i>	1.71	1.85	—	1.58	0.8 0.9	1.63	1.0
6	0.65	0.36	1.83	<i>1.45</i>	<i>1.45</i>	1.49	1.61	1.45	0.9 1.0	1.70	1.0
Mean \pm	0.61 \pm							1.45 \pm		1.69 \pm	
S.E.	0.02							0.04		0.014	

Shortest time from tension minimum during latency relaxation to separation 1.03 ± 0.04

Difference between shortest separation time at indirect stimulation and at direct stimulation 0.23 ± 0.03

All time values in milliseconds

Explanations

1 The animals are the same as those in table II B

2 The latency is measured from the start of the stimulating pulse to the earliest sign of latency relaxation

3 The time is measured from the earliest sign of latency relaxation to the lowest point of the curve

4 The times are measured from the earliest sign of latency relaxation to the point where the contraction curves for one impulse and for two impulses at the indicated intervals separate

The italics show values for each animal which do not differ by more than 0.03 msec from the accuracy of separate readings; the represented values being means of one run of increasing and one run of decreasing stimulation intervals

An analysis of the active state plateau duration after a single impulse

According to the method of Macpherson and Wilkie, the duration of the active state plateau after a single impulse can be determined by superimposing a contraction curve, in which the active state is prolonged at maximal level by a second impulse given early enough on a twitch contraction curve (Macpherson and Wilkie 1954, Norris 1961). The point where the contraction curves separate then indicates the end of the first active state plateau. According to Hill (1949), the contractile elements shorten against the series elastic elements during the ascent of an

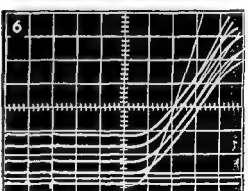
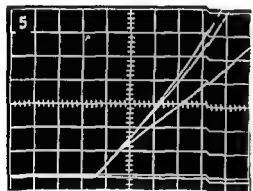
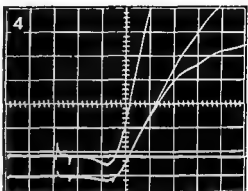
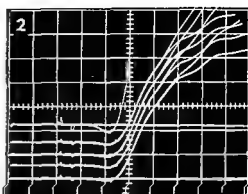
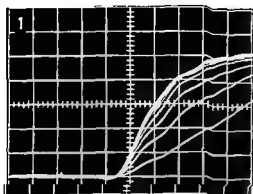
isometric contraction, and it will be considered here that the earliest obtainable separation point indicates the end of the active state plateau for shortening

The isometric contractions were recorded at high sensitivity, about 3/1000 of the peak tetanic tension per cm of the oscilloscope screen. To keep a fixed base line and to improve the resolution of the separation, the oscilloscope was connected via an RC high pass filter with a time constant of 1 msec. The contractions were recorded at the optimum muscle length for twitch peak tension.

As shown by Macpherson and Wilkie, the estimate is dependent upon the synchronism of activation. To get a synchronous activation, the starts of the twitches were recorded at the same amplification, sweep speed and filter time constants as was used for separation studies. The stimulation intensity was increased in steps and the start of the twitches were recorded for several steps on the same film (Fig. 1). The rate of tension development first increased for each step and the latency relaxation and the special form of the early phase of the contraction curve to be described later appeared more distinctly. After a certain stimulation intensity, determined for each preparation, the starts of the twitches became perfectly superimposed. This stimulation intensity was appreciably higher than that just supramaximal for twitch peak tension. The stimulation intensity used was somewhat higher than that just supramaximal for rate of tension rise.

In order to obtain a reliable measure of the active state plateau duration it is necessary to avoid repetitive firing for the first pulse. The absence of repetitive firing is indicated not only by the short plateau duration obtained but also by the constancy of twitch peak tensions (cf Ritchie 1954 a) for stimulation intensities from 20 to 30 V below to 20–30 V above the stimulation intensity used for synchronous activation (Range 150–170 V over the electrodes, 8 animals). To avoid repetitive firing, the pulse duration must be short (Gjone 1955). It is also necessary to prevent the high field intensity in the neighbourhood of a wire electrode in a volume conductor from reaching the muscle by placing the electrodes at some distance from it.

Table II A col. 4, gives for the separate animals the time intervals between the beginning of latency relaxation and the separation point of the contraction curves for direct single pulses and two pulses at different stimulation intervals. The values represent the means of one run of increasing and one run of decreasing stimulation intervals. A representative run is shown in Fig. 2. The italics in Table II A represent the time values which do not differ by more than the accuracy of 0.03 msec with which the readings were performed. If there is a plateau of active state initiated by the first impulse and if the second impulse, when it comes early enough, only prolongs the duration of this plateau, then there is a possibility that the time between the beginning of the latency relaxation and the separation point is constant for a range of stimulation intervals. As is evident from the results it has been possible to obtain such constancy in the experiments in spite of the difficulty of obtaining a full response for a second pulse at such short intervals. An interesting effect is always obtained at stimulation intervals shorter than 0.8 msec, where the contraction curve for paired stimuli first goes below the curve of the single stimulus, as shown in Fig. 3.



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Whatever the cause of this inhibiting effect on the first active state of an early second pulse, the effect shows that it is not possible to obtain shorter estimates of the plateau duration by this method. A similar effect was obtained by Brady, Abbott and Mommaerts (1961) in cardiac muscle for a train of pulses during the absolutely refractory period.

It may be further questioned whether there is a sufficiently long plateau of the active state, if any, or if the shortest time obtainable between the start of the first pulse and the separation point is limited by the sum of the irresponsive period and a mechanical latency for the second action potential (cf. Mårtensson and Sjöglund 1964). As shown in Fig. 4 and 5, the second impulse causes a prolongation of the initial course of the contractions if the separation occurs early enough. It is the contraction curve of a single impulse which deviates from the initial course after the separation. The same effect is also shown without filter distortion, with the oscilloscope DC-coupled, in Fig. 5. Here also contraction curves for two and three impulses are shown, where the same effect occurs once more, although it is less marked. In spite of a slowly declining active state, the later positive acceleration may possibly be largely ascribed to an increasing stiffness of the series elastic elements (cf. Sandow 1960). At no tension after the separation is the rate of tension development of the twitch as high as that of the contraction induced by two stimuli. This is not in accordance with the results of Close (1962).

One cannot escape the impression that the form of the contraction curves for one, two and three direct pulses shown here are consistent with the view that soon after a stimulating pulse there is a period of high and possibly approximately level activity which declines long before the twitch peak tension is reached, and that repeated stimulation can do no more than cause a prolongation of the initial high level of activity. It cannot be said, however, that this activity is exactly as high as that during a fully developed tetanus. This result has been obtained without any imposed disturbance of the muscle, as in Hill's experiments (1949, 1953) the interpretation of which has been criticized on this account by several authors (Goodall 1957, Pringle 1960, Walker 1960, cf. also Sandow 1960).

The obtained form of the contraction curves is probably not only due to the fidelity of the isometric recording system and the synchronous activation but also to small variations in properties in a dominant group of fibres.

The interpretation of the recorded form of the early phase of an isometric twitch presented here has to be compared with that of Sandow (1958, 1960) who suggests that the active state hump on his differentiated records indicates the termination of the development of the active state.

Fig. 5. Direct stimulation. Superimposed isometric contraction curves for one impulse and for two and three impulses at intervals of 0.9 msec. Time 1 msec/div. Ordinate about 1.5% of maximal isometric tension. Oscilloscope DC-coupled. Isometric contraction curves for single and paired 0.9 and 0.8 msec. The lowest curve shows the start of the stimulation artifacts there are triggering artifacts.

So much may be said about the end of the first active state plateau for shortening but when does it begin? In Fig. 2 and 4 the start of an isometric twitch is shown at about $2.5 \times$ greater amplification than that used for separation studies. The time after an impulse at which the latency relaxation begins is not influenced by the muscle length, as long as there is some resting tension, so that a drop in tension is evident (Sandow 1944; Abbott and Ritchie 1951). Hill (1951) has shown that an increase in the resistance to stretch occurs in the stimulated muscle, evidently at about the same moment as that at which the latency relaxation begins, and before there is any evidence of shortening. The time from the start of the stimulating pulse to the first evidence of relaxation will be arbitrarily described here as a period of mechanical latency, a period during which there is no sign of activity in the records. The values shown in Table II A, col. 2, are the shortest hitherto reported.

The values reported in Table II A, col. 5, for the time interval between the beginning of relaxation and the earliest obtainable separation cannot be regarded as reliable measurements of the plateau duration but they represent the largest possible values of the plateau duration after a single impulse about 1.5 msec. As is evident from recordings like Fig. 5 where also the duration of the second active state plateau may be estimated, the earliest active state plateaus are probably not much longer than about 1 msec.

The longest possible value for the duration of the active state plateau after a single impulse about 1.5 msec is considerably shorter than earlier estimates, even for mammalian muscles. The value reported by Norris (1961) for rat peroneus digiti quinti is about twice as long.

With indirect stimulation as is evident from the results in Table II A col. 7 the shortest obtainable times from the beginning of latency relaxation to the separation point are somewhat longer than those with direct stimulation. There are also no clear indications of any active state plateau in the records. A representative run is shown in Fig. 6. The asynchronous activation and the duration of the irresponsive period obviously interferes with the active state determinations in this case these effects however being slight for this muscle.

An analysis of the duration of the active state plateau during a fully developed isometric tetanus. According to the method of Ritchie (1954 b) the duration of the active state plateau may be estimated by determining the time between the last impulse during a just fused tension plateau and the beginning of relaxation after a correction for the period of mechanical latency. A similar estimate would be obtained from the stimulation interval just necessary for fusion if this could be determined exactly enough.

Determinations of this kind were made with the oscilloscope sweep triggered by one of the last impulses of a 150 msec pulse train of various frequencies. The oscilloscope was coupled through an RC high pass filter with a time constant of 10 msec which permitted records with a sensitivity of about 1/1 000 of maximal isometric tetanic tension (about 5 kp/cm of the oscilloscope screen without distortion of the rapid events studied). The sweep speed was 1 or 2 msec/div. As the stimulation

TABLE II ■ Results obtained from isometric contraction records at 150 msec of direct tetanic stimulation

Column 1 2									3	4	5
Animal number	Time from the start of the last stimulating pulse to relaxation at stimulation frequency								Empirical fusion frequency	Time from the last impulse at fus freq to start of relaxation	Time from the last impulse at fus freq to 1/1000 tension fall
	175	200	250	275	300	325	350	400			
1	54	59	39	37	40	37	—	—	300	40	63
2	58	48	39	41	35	37	36	30	325	37	61
3	47	—	—	—	33	34	34	34	350	34	69
4	44	46	46	41	41	42	41	44	300	41	70
6	48	48	44	42	42	40	42	24	300	42	64
Mean ±										39 ±	65 ±
S E										0.15	0.17

Column 6	7	8	9	10	11
Animal number	Time from an impulse to max rate of tension rise at 175 imp/sec	Difference between col 7 and col 4	Difference between col 4 and initial mech latency (Table II A)	Col 9 divided by the shortest sep time (Table II A)	Calculated fusion frequency according to col 9
1	27	13	33	23	300
2	25	12	30	22	330
3	30	04	29	20	340
4	30	11	35	26	290
6	26	16	35	25	290
Mean ±					
S E	28 ± 0.10	11 ± 0.20	32 ± 0.12	23 ± 0.12	310 ± 10

Time values in milliseconds. Frequencies in impulses/sec

artifact proved to give a convenient and exact measure of the time of the start of a pulse without chopping, it was not reduced. Measurements were performed at the length at which resting tension just disappears and a few determinations at the optimum muscle length for twitch peak tension. A difference of about 5 mm are also reported.

The measurements performed are indicated in Fig. 8. The results are given in Table IIB and a representative record of some of the measurements in Fig. 7. The

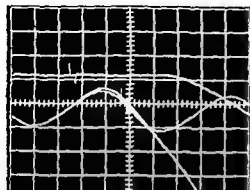


Fig 7 Direct stimulation Below Isometric oscillations obtained at 175 imp/sec for 150 msec. The sweep is triggered via a relay by the last pulse in one case and by the last but one in the other case. Latency is measured from the stimulation artifact to the time when the forms of the curves deviate from each other the records being read with a magnification of $5\times$. As the curves are

sec Sweep triggered by the last pulse but one. The fusion is almost complete. Filter time constant 10 msec. Time 1 msec/div. Ordinate 1/1000 of isom. max. tension (about 5 kp)/div.

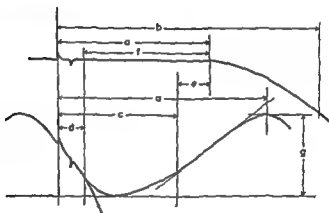


Fig 8

Above The events after the last impulse of a train at fusion frequency.

Below Oscillations during a not fully fused isometric tetanus with a relaxation curve after the last impulse superimposed.

The following measurements have been performed:

- The time from the start of an impulse to the beginning of relaxation.
- The time from the start of the last impulse to 1/1000 fall of tension.
- The time from the start of an impulse to the maximal rate of tension rise.
- Determination of the latent period.
- Oscillation amplitude.
- (e) and (f) have been calculated from the above determinations.

stimulation was direct. The time intervals measured did not vary by more than a very small percentage in the course of an experiment and were also approximately constant for an interval of stimulation intensity between the intensity which was used (180 to 200 V) and an intensity 30 V less.

Around the fusion frequency determinations were made in steps of 25 imp/sec. The frequency above that at which faint oscillations were still visible was arbitrarily described as the empirical fusion frequency (col 3). The time between the start of an impulse and the following peak or for frequencies above fusion frequency, to the

point where the curve began to deviate from the preceding level course, did not vary much around the empirical fusion frequency (col 2). According to Hill (1949), the recorded tension at the peaks, as well as during the level course, is just equal to the tension, P_0 , the contractile elements are able to bear when neither shortening nor lengthening. For frequencies below fusion, P_0 is declining at the peaks and the times in col 2 are generally longer the lower the frequency. A slight shortening of the times from pulse to peak is sometimes apparent at intermediate frequencies. This effect, also described by Cooper and Eccles (1930), is more marked at frequencies around 100 imp/sec, where a transient deepening of the oscillations occurs. These effects will be further described and analysed in a later paper. For frequencies above fusion, the last impulse might sometimes occur too early to cause a prolongation of the plateau for as long a time as that for lower frequencies. For frequencies higher than that for empirical fusion, a short and slight inhibiting effect after each pulse may be seen, an effect resembling that described in the preceding section. In order to obtain a good estimate of the duration of the active state plateau by Ritchie's method, it is necessary to select the right frequency.

In four experiments the muscle was stretched about 5 mm to the twitch optimum length. The determination of the time between the last pulse and the beginning of relaxation at the empirical fusion frequency then gave, in relation to that obtained at the shorter length, no change in one experiment and a prolongation of 0.1 msec in three experiments. This slight effect on the time to the estimated termination of the active state plateau may be an apparent one, caused by a retarding effect of the parallel elastic elements. No effects were evident on the form of the oscillations at lower frequencies.

The values for the time intervals from the last impulse at fusion frequency to relaxation (col 4) need a correction for mechanical latency to give an estimate of the duration of the active state plateau. In three special experiments a careful comparison was made between the form of the relaxation curve after the last impulse and earlier oscillations at a stimulation of 175 imp/sec (Fig 7). The mechanical latency from the start of an pulse to detectable positive deviation was found to be 0.1 msec longer than the time from the start of the first pulse to the beginning of latency relaxation. This difference is equal to the combined stationary state phase shift of the transducer and the high pass filter in the actual frequency range of sine wave components determined from the phase shifts introduced by filters with different time constants. There is thus no detectable difference between the initial mechanical latency and the latency after a 150 msec train at 175 imp/sec. The mechanical latency during a fused tetanus cannot, of course, be determined directly. The correctness of using the initial mechanical latency for the actual correction may, however, be estimated by subtracting the initial latency from the time interval between the start of the last impulse and the beginning of relaxation at the empirical fusion frequency. The times calculated in this manner should correspond to the stimulation interval just giving fusion. The fusion frequencies calculated in this way show good agreement with the empirical fusion frequencies (cols 11 and 3 respec-

tively) determined with frequency steps of 25 imp/sec. The values shown in col 9 with a mean (\pm the standard error of the mean, S.E.) of 3.2 ± 0.1 (5 animals) are then to be regarded as estimates of the duration of the plateau of the active state for tension maintenance according to Ritchie. The interpretation of these determinations will, however, be reconsidered in the discussion.

Two more kinds of measurement were performed on the records, the results of which will be further analysed in the discussion. The times from the last impulse at fusion frequency to 1/1,000 fall of tension are shown in col 5. The times from an impulse at 175 imp/sec to the attainment of the maximal rate of tension rise, measured in the way shown in Fig. 11 c, are given in col 7. A similar delay in the maximal rate of tension rise is evident for frequencies from 200 imp/sec up to the fusion frequency, although the oscillations cannot be analysed with the same accuracy. The time intervals between the attainment of the maximal rate of tension rise at 175 imp/sec and the start of relaxation at fusion frequency are shown in col 11.

As the experiments were performed on the same animals, a comparison in animals of the largest possible duration of the plateau of the active state for shortening after a single impulse and the duration of the plateau of the active state for tension maintenance at 150 msec stimulation at fusion frequency can be made. The ratios are shown in col 10.

No determinations of the kind described in this section were performed during indirect stimulation on these animals. In preliminary experiments the oscillations were of lower amplitude and more sine-wave shaped than those with direct stimulation and the records showed signs of repetitive firing after the last impulse. A larger hump after the preceding regular oscillations at frequencies below fusion generally occurred.

Discussion

The calculated fusion frequency of 310 ± 10 (S.E.) imp/sec in Table II B, col 11, is appreciably higher than the frequency of about 200 impulses/sec above which no substantial increase in isometric tension occurs. The values for the time interval between the last impulse at fusion frequency to 1/1,000 tension fall (Table II B, col 5) correspond, after a correction for mechanical latency, to a stimulation frequency of about 170 imp/sec. It has to be realized, however, that according to Hill (1919), the contractile elements are being stretched by the series elastic elements during the relaxation of an isometric contraction and that, according to Katz (1939), a muscle is able to resist considerable tensions with only a very slow lengthening. Presumably it is largely this great ability of muscle to maintain achieved tension even during the early phase of decline of the active state that enables the muscle to 'climb' up to almost maximal tension at 200 imp/sec, in spite of the stimulation interval being nearly 2 msec longer than the fusion interval that can be determined by a highly sensitive method. Owing to the steeper course of the force-velocity curve for shortening (Katz 1939), we must then expect the shortening velocity to be more

dependent upon stimulation frequency up to fusion frequency, than isometric peak tension. But the isotonic shortening velocity after 10 msec of stimulation increases with the frequency up to about 425 imp/sec, a frequency appreciably higher than the determined fusion frequency for the isometric tension plateau after 150 msec of stimulation. In line with this high optimal frequency, is the very brief duration of the plateau of the active state for shortening determined for the twitch, no longer than about 1.5 msec. The frequency of 425 imp/sec corresponds, however, to a stimulation interval of 2.35 msec. There may be some prolongation of the duration of the active state plateau, as shown by Ritchie and Wilkie (1955) for the later stages of the active state after previous stimulation, although the effect may be overestimated, owing to a difference in sensitivity of methods and a possible influence of refractoriness.

The estimated duration of the plateau of the active state for tension maintenance at 150 msec of stimulation at fusion frequency (Table II B, col. 9) of 3.2 ± 0.1 msec is considerable longer than the duration of the plateau of the active state for shortening after a single impulse. The maximal rate of tension rise after an impulse at a stimulation frequency somewhat below fusion, however, is not reached until near the end of the active state plateau, as estimated by the method of Ritchie. The differences are shown in Table II B, col. 8. In the tension region in which the oscillations occur, the fully activated muscle has a tension time curve which is convex upwards, as in the ordinary myogram. As long as the tension time curve is concave upwards, the muscle is not fully activated. The maximal rate of tension rise after an impulse indicates thus the beginning of the active state plateau for shortening (cf. Sandow 1960). It is evident that this active state is delayed in relation to that for shortening of a twitch. In the further analysis of these results two possible hypotheses that are independent, but do not exclude each other will be advanced.

Hypothesis I The active states for shortening and for tension maintenance do not run in parallel. This explains why there is such a discrepancy between the duration of the active state plateau estimated by Ritchie's method and the difference between the beginning of the active state plateau for shortening and the end of the active state plateau according to Ritchie's method. Further evidence is given by Hill's finding (1951) of a very early increase in resistance to stretch during the latent period which may be compared with the latency estimation at 175 impulses/sec described in the third section of the results. Support for this hypothesis is also given by the results of Jewell and Wilkie (1960) which show that during the relaxation of a twitch the tension at which the contractile elements neither shorten nor lengthen declines relatively more rapidly than the maximal velocity of shortening.

Hypothesis II The intensity of the active state cannot be increased above that existing during the plateau, but the activity caused by one impulse may summate with the activity caused by another impulse up to the limit set by the activity during a plateau. The maximal activity caused by one impulse is not developed abruptly and the activity during this rising phase may summate with the declining activity after the preceding impulse. When the impulse interval is short enough, fusion may occur, although the duration of the active state plateau for frequencies below fusion.

is still shorter than this interval. The duration of the active state plateau may even be as short as that of a twitch and the active state plateaus for shortening and for tension maintenance may coincide, although these phenomena are not necessary consequences of the second hypothesis. If the plateaus of the two active states coincide, however, Table II B, col. 8, gives an estimate of the duration of the active state plateau of 1.1 msec, about as short as that of a twitch, but the plateau is more delayed in relation to the impulse. Summation up to a certain limit, of the kind described above, may occur if a plateau of maximal activity is caused by a saturation of the contractile elements with an activating agent, the concentration of which is increased above that for saturation by each impulse and then decreases.

A well known explanation of the potentiation of a twitch is a prolongation of the active state. From the present work it appears that the rate of development of the active state is variable and this introduces a possible explanation of potentiating effects in which there is an increase in rate of tension rise and a shorter time to peak, as described, for example, for heart muscle (Brady *et al.* 1961) although the mechanisms of variation are not yet known. The results presented, however, also stress the importance of using a stimulus that is supramaximal for the event studied, e.g. rate of tension rise, and this must be especially important when the potentiating agent has an effect upon the excitability.

A smaller response for indirect stimulation than for direct, as obtained for isotonic shortening velocity, is classically attributed to a partial neuromuscular block. It is suggested here that another cause may also be the extinguishing of nerve impulses by collision, as synchronous muscle action potentials may re-excite the intramuscular nerve fibres (Lloyd 1942, Leksell 1945, Brown and Matthews 1960). If the time intervals involved are considered, it appears that possibilities of collision exist at high stimulation frequencies. The high optimal frequencies observed for indirect stimulation, which are not consistent with a predominating effect of Wedensky inhibition, may then be explained by more impulses reaching the muscle without collision when the stimulation frequency is increased. It should be noted here that with indirect stimulation there is a difference between a short stimulation interval and the first action potential interval in muscle (Eccles and O'Connor 1939). The records showed, however, that later action potential intervals followed the stimulation frequency sufficiently not to influence the interpretation of the responses to indirect stimulation.

Even if it is impossible for the nervous system to generate impulses at such short intervals as the duration of the plateau of the active state for shortening after a single impulse, the very short plateau duration may be of functional significance for brief corrections of movements when they tend to deviate from their intended course.

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Effect of Phenoxybenzamine and Hydergine on Urinary Catechol Amines in Rats during Restraint

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Abstract

GRAHAM, LINDSAY A. *Effect of phenoxybenzamine and Hydergine on urinary catechol amines in rats during restraint* Acta physiol. scand. 1966 68 18–22

output of A in urine during restraint

Physical restraint has been used for many years by Selye (1958) to produce stress in animals. It would be expected that the stress of muscular restraint would be associated with both increased neurogenic sympathetic and adrenomedullary activity.

This paper reports an initial investigation in which urinary adrenaline (A) and noradrenaline (NA) were measured in restrained rats. Furthermore, the sympathetic alpha-blocking agents phenoxybenzamine (PBA) and Hydergine were administered and the effect on urinary catechol amine excretion observed.

Materials and Methods

Male Sprague Dawley rats of about 150 g were used. Control (free) rats were kept in individual metabolism cages without food or water concurrently with restrained rats. Experimental rats were

Samples from free rats were paired those from restrained rats analyzed individually. Control of

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TABLE I Effect of PBA and atropine on the urinary catechol amine excretion rates ($\mu\text{g/kg/hr}$) of free and restrained rats. Numbers of samples in each group are in parentheses

		Saline	PBA 10 mg/kg	Atropine 15 mg/kg
Free	NA	0.12	0.75	
	A	0.03 (4)	0.17 (6)	
Restrained	NA	0.31	1.01	0.24
	A	0.28 (10)	0.51 (8)	0.28 (7)

Tests of differences between means for restrained rats

PBA vs Saline NA $p < 0.01$
 A $p < 0.05$

Atropine vs Saline NA NS
 A NS

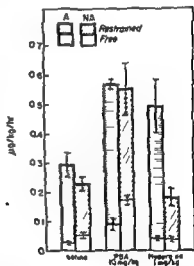


Fig. 1 Effect of PBA and Hydergine on free and restrained (16 hrs) rats. S.E. of mean is indicated for each group. Minimum $n = 6$, maximum $n = 10$.

Hydergine (Sandoz ■ A. Basle, Switzerland) and phenoxybenzamine as Dibenzylamine (Smith Kline & French) in injectable solution were given in single intramuscular injections in the leg immediately prior to restraint. Control animals were injected with equivalent volumes of saline and some restrained animals were given atropine sulphate. The order of drug injections was randomized.

Results

Effect of PBA and atropine on rats restrained for 12 hrs

Rats restrained for 12 hrs were injected with PBA 10 mg/kg. Atropine sulphate (15 mg/kg) was given to restrained rats to compare the effect of PBA with a vagal blocking agent. The effects of these drugs on urinary catechol amine excretion rates are shown in Table I.

TABLE II Effect of NA loading (100 μ g/kg) on the urinary excretion of NA (μ g/kg/hr) in PBA and Hydergine treated rats. Number of rats per group=8. Urine collection time 16 hrs

	Saline		PBA 10 mg/kg		Hydergine 1 mg/kg	
	A	NA	A	NA	A	NA
Control	0.03	0.06	0.09	0.17	0.05	0.04
NA loading	0.03	0.15	0.12	0.60	0.04	0.20
Recovery (%)		1.4%		6.9%		2.6%

There was an increased excretion of both A and NA during restraint, and a marked increase in NA in both free and restrained PBA-treated rats. Adrenaline excretion was also greater in these animals. There was no difference in excretion of either amine between control and atropine-treated groups. However, because of some difficulty with 12 hrs urine collections, the effect of PBA was repeated in rats restrained for 16 hrs.

Effect of PBA and Hydergine on rats restrained for 16 hrs

Free and restrained rats were given PBA 10 mg/kg or Hydergine 1 mg/kg. The effect of these drugs on the catechol amine excretion rates is shown in Fig. 1.

Restraint caused a significant increase in excretion of both amines. Furthermore, PBA caused an increased excretion of both amines, particularly NA, in free rats, while Hydergine-treated free rats were no different from controls. In restrained rats PBA caused a marked and significant increase in the excretion of both amines compared to restrained controls. The effect of Hydergine was to cause an increased A excretion with no change in NA excretion rate.

Effect of NA loading on PBA and Hydergine treated rats

The difference in NA excretion between PBA and Hydergine-treated rats could be due to greater release of NA at the nerve endings by PBA, or to inhibited destruction of released NA, perhaps due to blockage of "metabolic" as well as "effector" receptors. To test the latter hypothesis, a loading dose of NA (100 μ g/kg) was given to rats pre-treated by 1 hr with both drugs. Urine was collected for 16 hrs, with the animals in the free state. Recovery values were calculated on the basis of control values obtained from the same rats treated identically on the previous day, but without NA injections (Table II).

The recovery of 2.6% of the injected NA in Hydergine-treated animals compares with that of 1.4% for control animals. However, the 6.9% recovery found for PBA-treated rats suggests an interference in the inactivation of circulating NA by this drug.

Discussion

The increased A and NA excretion rates in restrained rats indicated that the stress of physical restraint caused marked increases in both adrenomedullary and neurogenic sympathetic activity. Thus the technique of restraining rats appeared to be a simple and effective way to study catechol amine metabolism during stress.

Phenoxylbenzamine caused an increased excretion of both amines in free rats, compared to controls. Schapiro (1958) has also found increased NA excretion with this drug, in similar dosages but found no change in A levels. It is possible that the difference is due to the chronic nature of Schapiro's study, compared to the acute values reported here.

With restraint the increase in the excretion of both amines observed in rats treated with PBA was still further accentuated. In the experiment with Hydergine, only the A excretion was increased. It seems likely that the effect of restraint compounded with the effect of these drugs caused a large adrenomedullary release of adrenaline. One reason for this may have been the general physical state of the animals since some, especially those treated with PBA, appeared to have lower body temperature and metabolic rates.

The increased NA excretion in restrained, PBA treated animals was possibly due to an interference with the re uptake of NA or inactivation of NA, either at metabolic sites near the nerve endings or in the liver, as suggested by Schapiro (1958). That such was, in part, the case was suggested by the greater excretion of an injected dose of NA in PBA treated animals. Such an effect did not occur with Hydergine. The possibility that PBA also caused a greater release of NA from the nerve endings cannot be excluded.

Both PBA and Hydergine have been shown by Brown and Gillespie (1957) to increase the output of NA from the spleen of the cat, when the splenic nerves are stimulated. These authors suggest that the blocking agent prevents the uptake by the splenic tissue of the liberated transmitter. Experiments by Blakeley, Brown and Ferry (1963) suggest that a proposed alternative hypothesis is unlikely, namely, that these adrenergic blocking agents act on a cholinergic link in the post ganglionic pathway (Boyd, Chang and Rand 1960). Furthermore at low frequencies of stimulation (less than 10/sec) the removal of liberated transmitter appeared to be complete (Brown and Gillespie 1957). Also Gillespie and Kirpekar (1963) have shown that when the cat spleen is perfused with NA PBA has a consistently greater capacity to prevent uptake of the infused NA into the spleen than does Hydergine.

It is likely, therefore that in rats pre treated with PBA recovery of infused NA was greater because of block by the drug of uptake at the nerve endings. The experiments of Gillespie and Kirpekar suggested that Hydergine would be less effective in this respect than PBA. That Hydergine appeared to have no effect at all may have been due to the dosage used. On the other hand since the animals were at rest the rate of spontaneous nerve stimulation may have been sufficiently low to allow similar uptake of both the endogenous and exogenous NA in the Hydergine tr

and control groups. Alternatively, different metabolic processes of NA might occur under the influence of the two drugs, with PBA being more effective in inhibiting the breakdown of NA in the liver, as suggested by Schapiro (1958).

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Surface Properties of Lung Extracts

I. A Dynamic Alveolar Model

By

FORREST H. ADAMS and GÖRAN ENHÖRNING¹

Received 29 August 1965

Abstract

ADAMS, F. H. and G. ENHÖRNING. *Surface properties of lung extracts I. A dynamic alveolar model*. Acta physiol. scand 1966 68: 23—27.

The importance of surface properties in the layer lining the alveoli is now well established. For measuring these properties a dynamic model of the alveolus has been constructed. It has the following advantages over previous techniques used to study the surface tension of biological fluids including lung extracts:

1. It more closely simulates the physiologic changes in rate and area that occur in the lungs during respiration.

2. Only a small volume of fluid (0.2 ml) is required.

3. The surface tension can be determined in 30 min or less.

It is suggested that the visco-elastic properties of the alveolar surface film may be important as well as the surface tension in determining the response to compression and expansion.

Over the past 10 years, there has been an increasing interest in the role of surface forces in the function of the lung in health and disease. Surface tension is a potential hindrance to lung expansion after birth and it tends to cause expanded alveoli to collapse again.

von Neergaard in 1929 suggested that the surface tension of alveoli might be lower than that of other body fluids due to accumulation of surface active material. He used the Lenard method for measuring surface tension which is similar to that of du Nouy (1926). These methods as well as the stalagnometric (Agostoni *et al.* 1958) are suitable for evaluating how surface tension resists lung expansion after birth when hemispherical air-liquid interfaces move down the airways. Recently this problem has also been studied with a modification of the maximal bubble pressure method which simulates the movement of an air-liquid interface through an airway (Enhörning 1964, Enhörning and Kirschbaum 1964).

Due to surface tension expanded alveoli tend to collapse but are protected by a lining layer. The significance of the ability of the lung lining layer to form stable

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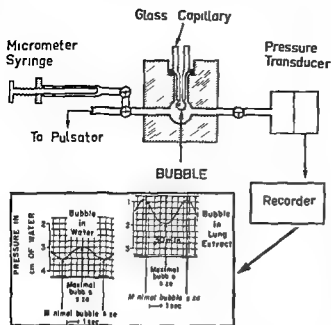


Fig. 1 Principle for studying surface properties with a dynamic alveolar model. A bubble communicates with atmosphere. Since bubble radius is measured through a microscope and since the pressure around the bubble is recorded, surface tension can be calculated. Bubble size is adjusted with micrometer syringe.

am was neglected until Pattle (1955) became interested in antifoam agents. He found that small bubbles expressed from cut lungs and observed through a microscope remained stable over 20 min. He thus concluded that the surface tension lining the bubble was very low, less than 0.06 dyn/cm . The method described by Pattle has been used successfully in an important clinical study (Pattle *et al.* 1962). However, it does not allow re-expansion of the film outlining the bubble and it is 'difficult to get reproducible results for the rate of contraction of bubbles' (Pattle 1965).

Many investigators studying the surface tension of the lung lining layer use the modified Wilhelmy balance (Clements, Brown and Johnson 1958). This instrument, however, requires a large volume of fluid, 50–70 ml, and the flat surface film developed in its trough cannot be compressed and expanded at the same rate as the alveolar film during respiration.

Since the importance of surface properties in the layer lining the alveoli is now well established, there is a need for a method which permits a fast evaluation of the surface properties and requires only a small sample volume. Furthermore the method should simulate the surface changes taking place in the alveolus during breathing. These points were taken into account when constructing a dynamic alveolar model for studying surface properties. Some experiences with this instrument have already been reported (Enhörning, Fujiwara and Adams 1964, Enhörning and Adams 1965) but a detailed description of the method is given in this paper.

Description of the apparatus

Fig. 1 presents the principal for studying surface properties with a model of an alveolus: a bubble which communicates with atmosphere and which can be made to change in size cyclically. A test chamber, with a volume of 0.2 ml containing the sample, communicates with the atmosphere via

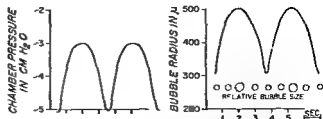


Fig 2 (Above) Tracing obtained when bubble was pulsating in water (Below) Since there was a constant surface tension of 72 dyn/cm, bubble radius could be calculated with Laplace formula

a vertical glass capillary. The chamber is also connected with a micrometer syringe, a pulsator unit, and a pressure transducer (Sanborn Model 268 A). The system is first completely filled with liquid

moved in and out of the system at a frequency that can be varied from 10 to 180 cycles/min. The

$= 2\gamma/r$ the surface tension of the sample (γ) can be calculated since ΔP and the radius (r) are known. In the formula ΔP is in dyn/cm², and consequently the value read from the record in cm of water is multiplied by 980. Since the radius is measured in μ but is in cm in the formula it is multiplied by 10^{-4} .

Surface properties as reflected by pressure recording

Fig 1 and 2 show pressure tracings obtained when a bubble is made to pulsate in water. The stroke

to pulsate in water or in saline solution, surface tension at the air-liquid interface remains at a constant value of approximately 72 dyn/cm, which is the surface tension of water at 20°C. Since surface tension of water is constant, the pressure tracing makes it possible to calculate how the bubble radius changes continuously during the pulsator cycle (Fig 2).

When the water in the test chamber is replaced with an extract from normal adult lung (Clements 1962), there is a reduced tension in the surface of the air bubble. This conclusion is drawn from the fact that the pressure gradient across the bubble surface is less even when the bubble is first expanded. During the following minutes while the bubble is maintained at a fixed radius, the surface tension becomes further reduced. This latter observation implies that in accordance with Gibbs' law, surface-active material is moving to the surface and thereby reduces the surface tension. With a bubble radius of 500 μ , the pressure gradient ΔP finally reaches a value of slightly less than 2 cm of water. From these figures, surface tension can be calculated to be approximately 40 dyn/cm. When the bubble is made to disappear and is replaced with a new one of equal size, the pressure gradient across the surface of this new bubble is again reduced according to the same pattern and reaches the final value of slightly less than 2 cm of water. When the pulsator unit is started, causing the bubble volume to oscillate, the pressure tracing is immediately conspicuously different from that obtained with water or saline solution and changes further with time (Fig 1). Whereas in water the product of the pressure gradient (ΔP) and the radius (r) remains constant, indicating a con-

chronously the product of these two entities which is proportional to surface tension γ oscillates greatly. By inserting corresponding values of ΔP and r into the formula of Laplace the maximal value of γ at maximal bubble size can be calculated as can the minimal value of γ which appears at minimal bubble size.

Discussion

The alveolar model described above confirms the observation made with the modified Wilhelmy balance (Clements *et al.* 1958) that area compression of a lung extract film results in a reduction of surface tension. A lesser reduction of surface tension can be correlated with conditions characterized by a tendency to develop atelectasis (Avery and Mead 1959, Gruenwald *et al.* 1962) an observation which may be given the following explanation.

At the end of expiration alveolar surface area is maximally compressed, since alveolar radius (r) is minimal at that time. From the formula of Laplace, $\Delta P = 2\gamma/r$ it is clear that if there is a substantially decreased surface tension (γ) when r is minimal the pressure gradient across the alveolar surface (ΔP), which is necessary for alveolar expansion, may also be minimal. However, with a lesser reduction of γ there may be no reduction but an increase of ΔP . To maintain alveolar expansion in case of an increased ΔP , intrathoracic pressure has to be more negative. At the end of expiration it is least negative and may not be sufficiently reduced to yield the necessary value of ΔP . As a result many alveoli, particularly the smallest, would tend to shrink and collapse whereas the remaining large alveoli would become even larger. This explains the typical histological picture of lungs from newborns dying of idiopathic respiratory distress: areas of atelectasis alternating with areas of emphysema (Gruenwald 1956, 1958). However, if γ decreases extensively during expiration, the ΔP required to maintain alveolar expansion would also decrease and would correspond to the existing negative intrathoracic pressure. Recording of pressure with the alveolar model demonstrates that as the alveolus becomes smaller the pressure gradient required to keep it expanded becomes less. This illustrates the stabilizing ability of the surface film.

It is of interest to note that the calculated surface tension at the interface of the bubble pulsating in lung extract oscillates around the value of surface tension at the interface of a non pulsating bubble. It would seem that the surface active material which becomes concentrated in the air liquid interface not only reduces surface tension, but it also forms a film with visco-elastic properties. This film offers resistance to any change in bubble size and when the pulsator unit moves liquid into the test chamber thereby forcing the bubble to become smaller, the surface film offers resistance to such a change. As a result there is an increase in pressure of the liquid surrounding the bubble. When the pulsator unit withdraws liquid from the test chamber, this is also resisted by the surface film. Consequently the negative pressure around the bubble becomes more negative than would be required to counteract the bubble shrinking action of surface tension alone. This interpretation implies that not only a reduced surface tension is required to maintain the alveolar stability but also other properties of the alveolar film may be of importance. To prevent collapse

of the recently expanded alveoli of the newborn infant, it might be of importance not only that surface tension is reduced quickly but also that other physical properties, such as viscosity and elasticity, develop rapidly

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Surface Properties of Lung Extracts

II. Comparison of Fetal and Adult Rabbits

By

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Abstract

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II Comparison of fetal and adult rabbits Acta physiol scand 1966 68 28
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compressed the tension decreased and when the surface area was expanded the tension increased. The difference between the mean value and the minimal or the maximal value was a function of change in surface area as well as time. The change in surface area and the rate with which it occurs in the lungs is more closely simulated with the dynamic alveolar model than with the modified Wilhelmy balance. The minimal value of surface tension was recorded at minimal surface area. It increased if surface area was maintained at minimal size. This demonstrates viscosity of the surface film. For alveolar stability the outlining film should probably have high viscosity as well as low surface tension. In the newborn these properties have to develop rapidly to prevent alveolar collapse.

The properties of the lung lining layer obtained by various methods appear to be important in health and disease (Pattle 1965, Avery and Said 1965). Pulmonary surfactant has been reported to appear during fetal life after 18 days in the mouse (Pattle 1961, Buckingham and Avery 1962) and 120 days in the sheep (Adams and Fujiwara 1963). In human fetuses it has only been found in those weighing more than 1 000 g (Avery and Mead 1959). Gruenwald, on the other hand, has shown that the lungs of some very young human fetuses possess high surface activity and display normal pressure volume curves (Gruenwald 1963).

The question remains, however, are the surface properties of lung extracts of term fetal animals identical with those obtained from normal adult animals? The results of the investigations to be reported indicate that the surface tension of lung

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TABLE I Surface tension (dyn/cm, mean \pm S D) in nonpulsating bubble as a function of time

	n	$\frac{1}{2}$ min	1 min	2 min	30 min
Fetal lung extract (3 g tissue/50 ml)	6	57 \pm 4	55 \pm 3	53 \pm 2	49 \pm 3
Adult lung extract (3 g tissue/50 ml)	8	48 \pm 6	47 \pm 6	45 \pm 7	41 \pm 5
Difference \pm S E		9 \pm 2.7	8 \pm 2.5	8 \pm 2.6	8 \pm 2.2

S D = Standard deviation S E = Standard error

extracts obtained from fetal rabbits at term is higher than that obtained from adult rabbits and that other properties of the lung lining layer may also be of importance

Material and Procedure

Eleven rabbits were used for this study. Six were pregnant and were within three days from term. All animals were sacrificed by intracasternal injection of 3–5 ml of 2% Xylocaine. The lungs were carefully removed for preparation of extracts which were then studied with the dynamic alveolar model previously described (Adams and Enhorning 1963) and with the modified Wilhelmy balance (Brown, Johnson and Clements 1959, Clements 1962). Of the extract the dynamic alveolar model required 0.2 ml and the trough of the modified Wilhelmy balance 50 ml. To obtain sufficient material the lungs from all fetuses of one litter were pooled. This gave 5 samples of fetal lung extract each large enough to be studied by both methods. However, one rabbit had only two fetuses and the extract in this case was sufficient only for a study with the dynamic alveolar model.

The extract from all babies of one litter was pooled and used for all studies.

For 1 ml of bubble = 1 g.

tracing indicated a clean chamber.

After calibration at -1 and -3 cm of water the saline solution was replaced with the unknown sample which was withdrawn with the micrometer syringe until a bubble with a radius of 500 μ was formed. While the bubble was maintained at this size the chamber pressure slowly became less negative. In a chamber of this size the pressure slowly became less negative.

Whenever a new bubble was formed its radius was then changed cyclically from a maximum of 500 μ to a minimum of 425 μ as the pulsator unit moved 0.2 mm³ of liquid in and out of the bubble chamber. For at least 30 min the pressure was recorded continuously while the pulsator was maintained at the same frequency and volume displacement. Cycling rates up to 180 min were also tested after which the pressure was recorded while the bubble radius was maintained stationary at 500 μ .

In this study the minimal value of surface tension for a given sample was defined as the lowest value obtained during a 16 hour recording and the maximal value as the highest value noted during this time.

Ten extracts of adult lungs (8 of normal concentration, 2 of higher concentration) and 5 extracts of fetal lungs were studied by both methods. The extract of the lungs from the eleventh rabbit was examined in a different manner using the Wilhelmy balance. The surface area was not only compressed to 20% of its maximal size but also to approximately 40%, 60%, and 80%.

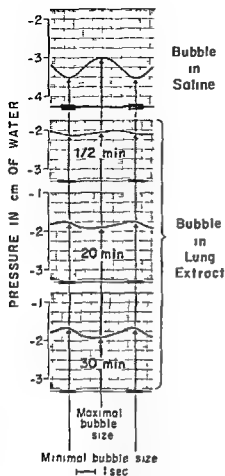


Fig 1

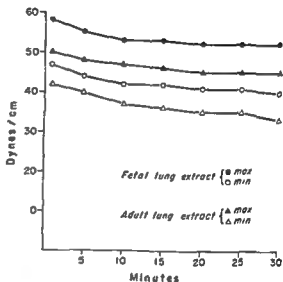


Fig 2

Fig 1 Pressure tracing obtained when bubble was pulsating in fetal lung extract

Fig 2 Mean surface tensions at maximal and at minimal bubble size showing progressive decrease with time

Results

Table I contains the results of the surface tension measurement as a function of time in a nonpulsating bubble from fetal and adult lung extracts. The mean fetal values obtained at 1, 2, 1 and 2 min after formation of the bubble and after 30 min pulsation were significantly higher ($P < 0.01$) than the corresponding extracts from adult lung.

When the pulsator unit was turned on, a very characteristic pressure tracing was obtained with saline solution in the sample chamber. The pressure was most negative (ΔP_{max}) at the time when the bubble was smallest.

As soon as the saline solution had been replaced with lung extract the pattern of the pressure tracing was different (Fig 1). The most negative pressure no longer coincided with minimal bubble size. Instead, it occurred while the bubble was

TABLE II Surface tension (dyn/cm² mean \pm S.E.) in pulsating bubble as a function of time

	n	1 min		5 min		10 min	
		max	min	max	min	max	min
Fetal lung extract (3 \square tissue/50 ml)	6	58 \pm 2.3	47 \pm 1.7	55 \pm 3.8	44 \pm 1.4	53 \pm 2.4	42 \pm 2.0
Adult lung extract (3 \square tissue/50 ml)	8	50 \pm 4.2	47 \pm 2.9	48 \pm 4.7	40 \pm 4.4	47 \pm 4.3	37 \pm 4.2
Difference \pm S.E.		8 \pm 1.8	5 \pm 1.2	7 \pm 2.7	4 \pm 1.7	6 \pm 2.0	5 \pm 1.7

\square = Standard deviation \square E = Standard error

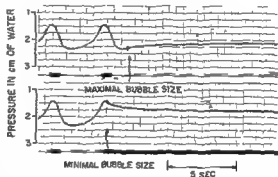


Fig 3 Tracings obtained when pulsator was stopped at maximal and minimal bubble size showing spontaneous change in pressure toward the mean

becoming larger. The tracing of a newly formed bubble, pulsating in lung extract was slightly skewed, but with continued recording it became more symmetrical, and the pattern finally became almost the inverse of that of saline, i.e. the most negative pressure (ΔP_{\max}) preceded and almost coincided with maximal bubble size. Thus, the apparent value of surface tension was minimal when the bubble was smallest and maximal when the bubble was largest. These extreme surface tension values for a pulsating bubble were above and below that obtained with a nonpulsating bubble. The maximal and particularly, the minimal apparent surface tension decreased as a function of time as seen in Table II and Fig 1 and 2. The fetal values remained higher than those of the adult, and the difference between them after 30 min of pulsating was highly significant ($P < 0.01$).

When the speed of the pulsator unit was increased there was no change in maximal or minimal pressure with saline solution in the sample chamber but with a lung extract there was an increase in pressure amplitude. With an extract of fetal origin and with a frequency change from 17 to 180 cycles/min the amplitude increased by $69 \pm 23\%$ (mean \pm S.D.). With an extract of adult lungs the increase was $46 \pm 5\%$ (mean \pm S.D.) with the same frequency change. The difference between the two lung extracts was 23% with a standard error of 10% . The amplitude increase affected maximal and minimal pressure values equally. When the pulsator

15 min		20 min		25 min		30 min	
max	min	max	min	max	min	max	min
53±2.5	42±1.8	52±2.4	41±2.3	52±2.7	41±2.4	52±2.5	40±3.2
46±4.3	36±4.0	45±3.8	35±3.5	45±3.2	35±3.8	45±3.3	33±4.2
7±1.5	6±1.5	7±1.7	6±1.5	7±1.6	6±1.7	7±1.6	7±1.9

TABLE III Surface tension (dyn/cm, mean±S.D.) as it appears when surface area is compressed and expanded in trough of Cap. Wilhelmy balance and in pulsating bubble

	Fetal lung extract (3 g tissue/50 ml)		Adult lung extract (3 g tissue/50 ml)	
	max	min	max	min
Wilhelmy balance	69±3.4 n=5	24±3.5 n=5	47±7.4 n=8	12±1.5 n=8
Bubble chamber	52±2.5 n=6	40±3.2 n=6	45±3.3 n=8	33±4.2 n=8

S.D. Standard deviation.

unit was turned off, either at maximal or at minimal bubble size, the apparent surface tension tended to approach the mean value as shown in Fig. 3.

The results thus far given were obtained with the extracts prepared with 3 g lung tissue per 50 ml of saline solution. With the more concentrated extracts (6 g tissue/50 ml), prepared from the lungs of 2 adult rabbits the tracings showed the same pattern but the values for apparent surface tension at minimal bubble size decreased faster and were lower. After 30 min of pulsation, these values for the two rabbits were 29 dyn/cm, and 28 dyn/cm respectively.

With the Wilhelmy balance the minimal value of surface tension in fetal lung extract was 24 ± 3.5 dyn/cm (mean ± S.D.) whereas in adult lung extract the value came down to 12 ± 1.5 dyn/cm (mean ± S.D.). The difference 12 dyn/cm has a standard error of less than 1 dyn/cm and is highly significant ($P < 0.01$).

As seen in Table III the minimal values recorded with the modified Wilhelmy balance were lower and the maximal values higher than those obtained with the dynamic alveolar model. However, when there was less reduction of the surface area in the trough of the Wilhelmy balance, the minimal value increased and the maxi-

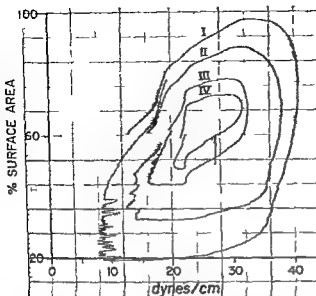


Fig 4

Fig 4 Tracing obtained with modified Wilhelmy balance when surface area was reduced to 20% (I) 40% (II) 60% (III) and 80% (IV) of original size. Results show effects of change in film compression but may also show effects of aging since IV was made first and I last.

Fig 5 Maxwell element

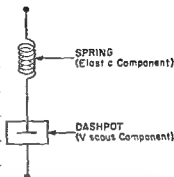


Fig 5

mal value decreased so that the result became more similar to that obtained with the dynamic alveolar model (Fig 4)

Discussion

Difference between adult and fetal lungs With both methods used in this study it was found that the extracts showed more surface activity when they originated from adult than from fetal rabbit lungs. This probably reflects a difference in concentration of surface active material since more concentrated extracts prepared from 6 g instead of 3 g tissue per 50 ml yielded lower values of surface tension at minimal surface area. However in view of the demonstration by Levine and Johnson (1964) that surface active material is more readily extracted from aerated than from atelectatic lungs it would seem unsafe to conclude that also in lungs of adult rabbits is there a higher concentration of surface active material. Nevertheless there is reason to believe that aeration of the lungs after birth will promote a concentration of surfactants in all the air liquid interfaces produced in the alveoli. Such a movement of surfactants to the surface would be in accordance with Gibbs law and is demonstrated to occur in the dynamic alveolar model by the increase in pressure amplitude during the 30 min of recording (Fig 1). Thus as the surfactants are being produced by alveolar cells or as they arrive to the lungs by way of the blood stream they will have a tendency to remain in the air liquid interface and their concentration if insufficient at birth may finally become adequate. This might explain why in

many cases of respiratory distress in the newborn there is a spontaneous regress of the symptoms

Influence of surface compression on minimal value of surface tension The very low value of surface tension characteristic for a normal lung extract is recorded with the modified Wilhelmy balance when the surface film is compressed to approximately 20 % of its maximal size. With less compression the value is higher (Fig. 4). If this is analogous to *in vivo* conditions the very low value of surface tension is reached only if the air-liquid interface of the alveolus is compressed to 20 % of its maximal size. Yet according to Pattle it is conceivable that respiration causes no surface area reduction. He visualizes the alveolus as a wrinkled paper bag which may be blown up without any change of its inner surface area (Pattle 1963). Usually, however, the alveolus is thought of as a spherical bubble blown at the end of a narrow tubing.

Assuming the alveolus has a spherical shape it can be calculated how breathing changes its surface. In a young healthy man the tidal volume is about 600 ml and the functional residual capacity 2400 ml (Comroe *et al.* 1962). The volume at the end of normal expiration is therefore 4/5 of that at the end of normal inspiration. Since the volume of a sphere is proportional to the third power of its radius the alveolar radius at the end of expiration will be what it is at the end of inspiration multiplied by $\sqrt[3]{4/5}$. The surface of a sphere is proportional to the square of its radius and therefore the alveolar surface at the end of expiration will be what it is

at the end of inspiration multiplied by $[\sqrt[3]{4/5}]^2$. Thus at the end of expiration the surface area has been reduced to 86 % of what it was at the end of a normal inspiration. With deeper breathing the reduction in surface area may be greater but even at maximal expiration in young healthy men the surface area is compressed only to 34 % of its maximal size. This figure is derived by assuming that the residual volume is 20 % of the total lung capacity. However in men 50 to 60 years old residual volume is 40 % of total lung capacity (Comroe *et al.* 1962) and under those circumstances the maximal surface area reduction will be 54 %. These calculations are in agreement with those of Storey and Staub (1962). They measured diameters of alveoli in rapid frozen cat lungs and calculated that the surface area could be increased 70 %, which means that the minimal area is 60 % of the maximal. In view of these estimations of how the alveolar surface area changes during breathing the surface of the alveolus in the dynamic model at end of expiration was chosen to be approximately 70 % of its maximal size.

With normal breathing the changes in alveolar surface area are hence quite small and the true values of surface tension are probably not as high as the maximal and not as low as the minimal values recorded with the Wilhelmy balance. The true values oscillate around a mean obtained when there is no change in surface area. The film outlining the alveolus lowers surface tension but it also seems to resist any change in size. To keep the alveolus expanded in the lung extract a negative pressure had to be exerted. But when the alveolus was made smaller (expiration) the required negative pressure decreased as if the surface film opposed

the change and to a certain extent neutralized the effect of surface tension. When on the other hand the "alveolus" was made larger (inspiration) the surface film seemed to resist this change too. Therefore a greater negative pressure had to be exerted than would be needed to overcome the effect of surface tension alone.

Influence of cycling frequency on minimal value of surface tension The minimal value of surface tension, recorded during maximal reduction of surface area, is a function not only of this area but also of time. This observation confirms what has previously been reported (Sutnick and Soloff 1963). If the area is maintained at minimal size the recorded value of γ will increase, and if maintained at maximal size it will decrease. When a lung extract was examined with the alveolar model the pressure amplitude became greater with increasing rate of pulsation. As originally pointed out by Avery and Mead (1959) it is as if visco-elastic properties had been formed in a surface film.

A biological visco elasticity may be illustrated with a Maxwell element which consists of a spring, representing the elastic component, in series with a dashpot, representing the viscous component (Stacy *et al* 1955) (Fig 5). A dashpot consists of a container with oil in which a disc moves in a direction which is perpendicular to its surface. If there is a sudden increase in length of the Maxwell element, this will be taken up entirely by the spring, since the dashpot requires time to move. According to Hooke's law the increase in tension is proportional to the increase in length of any element which is elastic. However, with time, the dashpot will start moving, and this will diminish the stretching of the spring and thus its tension. The dashpot will move more slowly, the decrease in tension being exponential. The higher the viscosity of the viscous component, the longer it takes for the spring action to be reduced.

When alveolar pulsations were suddenly stopped at maximal or minimal "alveolar" size or when the barrier of the modified Wilhelmy balance was stopped at maximal or at minimal surface area, there was an apparent exponential decrease in stretching of the elastic surface film component (Fig 3 of this article and Fig 6 of paper by Sutnick and Soloff 1963). This decrease in stretching would go faster with low viscosity in the surface film. The increase in pressure amplitude with increased cycling frequency of the alveolar model suggested that at the low frequency of 17 cpm there was time to allow a reduced stretching and compression of the elastic surface film component as the bubble approached maximal and minimal size, respectively. Thus, the increase in pressure amplitude observed particularly with the extracts of fetal origin would seem to reflect low viscosity of the film outlining the bubble. At low frequency the viscous component, "the dashpot" had time to move and the stretching of "the spring" was reduced.

Breath holding at maximal expiration could jeopardize alveolar expansion if viscosity of the alveolar film is low. This would allow rapid disappearance of the spring action, i.e. a fast increase of surface tension. Thus it would seem conceivable that for alveolar stability the film outlining the alveolus should have not only low surface tension but also high viscosity. In the mature fetus these properties

Surface Properties of Lung Extracts
III. Changes Appearing during First Ten Minutes
after Surface Formation

By

FORREST H ADAMS, GÖRAN ENHÖRNING¹ and MAUD REGNE-KARLSSON²

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Abstract

ADAMS, F H, G ENHÖRNING and M REGNE-KARLSSON. *Surface properties of lung extracts. III. Changes appearing during first ten minutes after surface formation* Acta physiol scand 1966 68 37—42

Surface properties of washings and extracts from fetal guinea pig lungs were investigated with the dynamic method of Adamson (1959).

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time. After 4 min the mean values were significantly higher in lung extracts from fetuses weighing more than 60 g than in lung extracts from fetuses weighing less. — Thus after only 2 min of measurement and with a small sample (0.2 ml) the method makes it possible to differentiate immature from more mature lung surface properties. The method might also make it possible to differentiate normal infants from those who subsequently develop idiopathic respiratory distress by examining the fluid obtained by aspiration of the upper airways.

Idiopathic respiratory distress of the newborn is still a major cause of neonatal mortality (Ahvenainen 1959, Arcy 1960, Avery and Oppenheimer 1960). A prominent feature of the condition is collapse of many alveoli (Gruenwald 1956, Briggs and Rigg 1958). For alveolar stability, surface tension has to decrease extensively during expiration and increase during inspiration (Brown, Johnson and Clements 1959), and according to Avery and Mead (1959) and Patle *et al.* (1962) this important surface property is deficient in most infants dying from idiopathic respiratory distress. To prevent alveolar collapse the surface properties must develop rapidly, probably within the first few minutes after initial aeration.

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TABLE 1 Surface tension (dyn/cm) in a nonpulsating bubble in lung extract as a function of time (mean \pm S.D.)

Group no	Weight	n of subjects	1 min	2 min	10 min
1	<60 g	17	49.2 \pm 4.0	47.4 \pm 4.0	33.4 \pm 4.0
2	>60 g	21	46.8 \pm 4.2	41.8 \pm 4.2	33.1 \pm 4.0
1+2	3.4-135 g	38	47.9 \pm 2.9	44.2 \pm 3.1	33.3 \pm 2.9
Difference between groups 1 and 2 (D \pm S.E.)			2.4 \pm 2.6	5.6 \pm 2.6	0.3 \pm 2.6

S.D. = Standard deviation S.E. = Standard error

This paper concerns the surface properties of washings and extracts from fetal guinea pig lungs determined with the dynamic alveolar model previously described (Adams and Enhorning 1966). Special attention has been given to the early changes in the air-liquid interface and to the correlation of these changes with fetal weight.

Materials and Methods

fetuses were lost due to technical difficulties.

The pregnant guinea pigs were sacrificed by intracisternal injection of 2-5 ml of 2% Xylocaine. Immediate laparotomy was performed and before delivering the fetuses their necks were ligated to prevent aspiration of amniotic fluid. Fetal weight was recorded in each case.

For this study the pulsator of the dynamic alveolar model was adjusted to alter the "alveolar

formed. The bubble was maintained at this size for 2-3 min. It was then collapsed and replaced with a new bubble which pulsated at 17 cpm for at least 10 min. Finally the pulsator was stopped and the bubble was made to remain stationary at a radius of 500 μ for an additional 2-3 min.

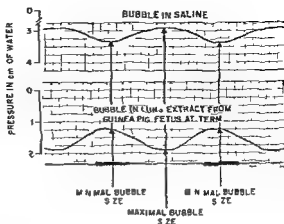
1966)

As a way of expressing the stabilizing ability of the surface film, the stability index \bar{S} was calculated using the formula

$$\bar{S} = \frac{2(\max - \min)}{\max + \min} \quad (\text{Clements et al. 1961})$$

sure coincided with the mark in sample the most negative pressure = which reflects viscosity was ex = was 360 degrees

Fig 1 Pressure tracings obtained when bubble pulsated in saline and in lung extract. In both liquids bubble radius changed from a maximum of 500μ to a minimum of 450μ



Results

With a nonpulsating bubble in lung washings, surface tension (mean \pm S D) after 1, 2 and more than 10 min was 61.4 ± 2.6 , 59.9 ± 2.2 , and 56.2 ± 3.5 dyn/cm respectively. The values for lung washings were high for both mature and immature fetuses but for lung extracts they were lower (Table I). The surface tension decreased faster when the extract was derived from fetuses weighing more than 60 g.

With a pulsating bubble in lung washing or lung extract, the most negative chamber pressure, thus the maximal value of ΔP , no longer coincided with minimal bubble size as it did when there was saline in the test chamber (Fig 1). Instead ΔP_{max} occurred while the bubble was becoming larger. The phase difference was apparent already at beginning of bubble pulsation but increased with duration of cycling and finally approached 180° . Thus after 10 min surface tension decreased as the bubble became smaller, and increased as the bubble became larger. Since pressure amplitude as well as phase difference increased with time, the difference between the maximal and the minimal value of surface tension also increased as a function of time. After 10 min the maximal and minimal value of surface tension (mean \pm S D) was 61.0 ± 2.6 and 46.4 ± 2.9 dyn/cm in washings and 46.4 ± 3.5 and 27.9 ± 3.7 dyn/cm in extracts.

The decrease of the maximal and particularly the minimal value of surface tension during the 10 min of recording is reflected in an increase of stability index. After 2 min the mean value for lung washings was 0.24 ± 0.02 and after 10 min 0.27 ± 0.03 . The 2 min value of lung washings did not change with fetal weight as did the value of lung extracts. Two minutes after bubble formation the stability index was still below 0.30 in each of the extracts from fetuses weighing less than 60 g but was above this value in extracts from 13 of the 21 fetuses weighing more than 60 g (Fig 2). Table II shows the increase in the 2 min value of stability index with increase in fetal weight. The differences between the values of group 2 and 3 of this table (0.22) has a standard error of 0.07 and is significant $P < 0.01$.

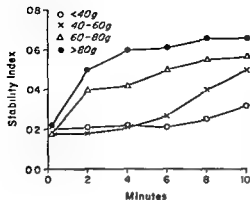


Fig 2 Change of stability index with time. Mean values for fetuses in different weight groups. Already after 2 min the index was significantly higher for fetuses weighing more than 60 g than for those weighing less.

TABLE II Stability index and phase difference two minutes after bubble formation in lung extracts

Group no	Fetal weight	n of subjects	\bar{S} (mean \pm S D)	Phase difference in degrees (mean \pm S D)
1	<40 g	8	0.21 \pm 0.04	125 \pm 4.6
2	40-60 g	9	0.18 \pm 0.07	120 \pm 6.2
3	60-80 g	9	0.40 \pm 0.15	150 \pm 16.5
4	>80 g	12	0.45 \pm 0.15	150 \pm 13.2
Difference between groups 2 and 3 (D \pm S E)			0.22 \pm 0.07	30 \pm 8

S D - Standard deviation S E - Standard error \bar{S} Stability index

Phase difference increased slowly in lung washings but rapidly in lung extracts. Two minutes after bubble formation, it had the same value in lung washings from fetuses weighing less than 60 g as from those weighing more. In lung extracts from 12 of 21 fetuses weighing more than 60 g, the 2 min value of phase difference had become greater than 140°, but in none of the extracts from fetuses weighing less than 60 g had the phase difference reached this value. Table II demonstrates how the phase difference after 2 min was greater in lung extracts from the heavier fetuses. The difference between groups 2 and 3 (30°) has a standard error of 8° and is significant ($P < 0.01$).

Discussion

The results obtained in this study indicate that lung extracts contain more surface active material than do airway washings. In view of previous studies (Levine and Johnson 1964, Enhörning, Fujiwara and Adams 1964, Adams *et al.* 1965) it is likely that the higher minimal value of surface tension, observed in the washings, is due to a low concentration of active phospholipids rather than to a change in their composition. With the technique used for washing the airways, there might have been an

incomplete removal of surface active material from the alveoli. In the fetal lamb there is a continuous flow of fluid out through the trachea (Adams, Moss and Fagan 1963) and when this fluid was studied with the dynamic alveolar model it was found to have high surface activity (Enhörning and Adams 1965). A study of the undiluted airway liquid would have been preferable but with the present design of the apparatus it does not permit examinations of the small volumes of liquid obtainable from the airways of fetal guinea pigs.

The observation that the lungs from more mature fetuses yielded a film with greater stabilizing ability is in agreement with previous studies in other species (Avery and Mead 1959, Buckingham and Avery 1962). The close relationship between stability index and phase difference (Table II) should be noted. It is conceivable that the increase in both these entities during the 10 min recording is due to an increase in viscosity of the bubble's surface layer. Langmuir observed the existence of viscosity in a surface film and described a method for its determination (Langmuir 1917). It was suggested by Avery and Mead (1959) that the surface film of a lung extract has both viscous and elastic entities. Adams, Enhörning and Norman (1966) have discussed these properties further and have come to the conclusion that alveolar stability may be jeopardized if viscosity of the outlining film is low. Recording of the pressure gradient across the surface of a pulsating bubble indicates that, due to visco-elastic properties of the outlining film, it resists any change in bubble size. When the radius is maintained at a fixed value, the liquid must have a certain negative pressure to overcome the effect of surface tension. If the bubble is made larger by withdrawing some of the liquid surrounding it, the pressure in the liquid becomes more negative since the tension in the film increases with stretching. When the bubble is made smaller, the surface film resists this change also and the pressure in the liquid surrounding the bubble becomes less negative. If the viscosity of the surface film is high, the film will yield very slowly to the stress it is subjected to during pulsation. The most negative pressure will then coincide with the largest size of the bubble and the least negative pressure with the smallest bubble size. However, the lower the viscosity of the surface film, the faster it will yield to a stress. The change of bubble size during pulsation is sinusoidal. Thus as the bubble approaches a maximum or a minimum, the rate of change in surface area becomes slower. With low viscosity there will then be time for the surface film to relax prior to maximal stretching or compression. However, the higher the viscosity of the surface film the greater is the difference in phase and also the pressure amplitude and thus the stability index will be greater. Hence the important property of the film lining the alveolus is not limited to its ability to reduce surface tension. It may be of equal importance to alveolar stability that the film has the correct visco-elasticity which develops rapidly after initial aeration.

The present findings indicate that extracts of immature and mature fetuses can be differentiated by a 2 min pressure recording. With the airway washings which were greatly diluted, this was not possible. However, the undiluted upper airway fluid of normal newborns may be different from that of infants who subsequently

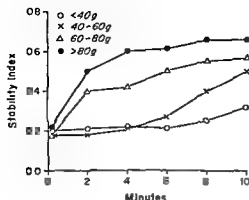


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S D = Standard deviation S E = Standard error \bar{S} = Stability index

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Body Protein and Fat, Blood Glucose and Free Fatty Acids in Various Strains of Mice

By

STIG LARSSON

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Abstract

LARSSON, S. *Body protein and fat, blood glucose and free fatty acids in various strains of mice*
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Carcass composition was determined in the NMRI, CBA Swiss and DBA strains and in NMRI Swiss made obese by injection of goldthioglucose. Fasting for 24 hrs resulted in different degrees of weight loss depending on the strain. At the same time analysis of carcass composition revealed strain differences with regard to protein and fat. The changes of glucose and FFA in plasma after 24 hrs without food also varied among the strains.

Food intake is considered to be regulated by the central nervous system, viz the diencephalon. By lesions in the ventromedial parts of the hypothalamus, the satiety part of the feeding centre is destroyed which leads to an increase in food intake, eventually resulting in obesity (Brobeck 1955). For the normal regulation of food intake these parts of the diencephalon need to be intact. On the other hand, spontaneous obesity can occur without any detectable damage of the central nervous system (cf Mayer 1963). The etiology of some of these types of obesity has been intensively studied and partly elucidated. In certain types of adipositas, overweight is not found but a relative increase of body fat with age. Such data have been given for animals (Moulton 1923) and men (Brozek 1952, Fenton and Dowling 1953). We have studied this problem in mice and found that the excessive carcass fat deposition was accelerated by giving the mice a diet high in fat. In previous studies in pigs (Larsson, Nilsson and Olsson 1965) and in rats (Larsson 1965) the biological value of the protein of the diet was found to be of great importance for the fat deposition of the body and suggested to exert some influence with regard to obesity. The present experiments were undertaken to study the problems related to obesity in mice in relation to different strains of mice viz the carcass composition, blood glucose and free fatty acids (FFA).

TABLE 1 The effect of 24 hrs fasting on body weight in different strains of mice

n = 11 except for VMRI \times Swisse where n = 5

Strain	Decrease in weight % of prestarving wt
VMRI	12.2 (10.1-14.2)
CBA	13.7 (12.2-15.6)
Swisse	21.3 (17.9-24.1)
DBA	16.6 (14.3-18.7)
VMRI \times Swisse (obese)	15.1 (13.2-18.3)

Experimental

Male mice of the VMRI, CBA, Swisse, and DBA strains were used. They were all fed *ad libitum* on a fortified laboratory diet used for breeding (AB Teknosan, Sweden) which contained large amounts of animal proteins and about 3% fat. The protein content was 22.6%. All animals had free access to tap water.

Experiment 1

Eighteen mice of each strain were given the diet for 90 days. At this time they were all starved for 24 hrs and the change of body weight during the starvation period measured.

At the same time 18 other mice of the different strains were kept under the same conditions except that they were not starved on the 90th day.

All mice were sacrificed on the 91st day and the bodies of each mouse subjected to individual carcass analysis as has been described earlier (Larsson 1963). Thus values for total body protein and fat were obtained.

Experiment 2

Ten obese mice were obtained by injecting goldthioglucose (1.2 mg/g) (Scheering, Germany) into 10 mice VMRI \times Swisse after 24 hrs starving. At the time of injection the animals were 65-80 days old. The animals were not used until they had reached a constant weight level on the particular

*Experiment 1**Experiment 3*

On 20 animals of each of the different strains, blood samples were taken at 85 days of age. From here on samples were taken once a week either in the fed or starved state. The blood was analyzed as described above with regard to glucose and FFA. In order to obtain enough blood for the FFA determinations, samples from 4 animals were pooled.

Results*Experiment 1*

As may be seen from Table 1 the weight loss after 24 hrs without food was different depending on the strain of mice.

The Swisse mice showed the most marked weight loss. Table II gives the carcass contents of protein and fat of the mice from the different strains. The total protein varies comparatively little in the fed state while the starved animals show definite strain differences. Thus the VMRI strain shows no relative decrease in body protein after 24 hrs without food. The Swisse mice have a pronounced decrease in this respect. Starving decreases fat content in all strains but to different degrees. Table

TABLE II The effect of 24 hrs fasting on carcass contents of protein and fat in different strains of mice

F=fed mice S=standard mice $n=18$ except for NMRI \times Swisse, where $n=5$
 Differences calculated according to Student's *t* test

Strain		Protein % of body wt	Fat % of dry body wt	Prot/Fat
NMRI	F	19.1 ± 0.46	32.5 ± 0.98	1.8
	S	20.1 ± 0.43	26.1 ± 0.73^1	2.3
CBA	F	17.3 ± 0.82	36.9 ± 0.82	1.2
	S	15.2 ± 1.23	22.9 ± 0.91	1.9
Swisse	F	18.8 ± 0.72	23.0 ± 1.10	2.7
	S	13.1 ± 1.12^1	12.7 ± 0.83	3.4
DBA	F	19.3 ± 1.21	29.6 ± 0.88	2.7
	S	16.6 ± 1.33	15.6 ± 0.90^1	2.4
NMRI \times Swisse (obese)	F	13.4 ± 0.78	68.5 ± 1.63	0.3
	S	13.3 ± 1.26	61.1 ± 1.13^1	0.5

¹ $P < 0.001$, ² $P < 0.01$, ³ $P < 0.05$

TABLE III The effect of 24 hrs fasting on plasma content of glucose and FFA in different strains of mice

Glucose $n=20$ (obese=10) FFA $n=5$ repeated at 4 occasions (obese=10 repeated at 4 occasions) Differences calculated as in Table II

Strain	Decrease in blood glucose %	FFA in plasma $\mu\text{eq/ml}$	
		Fed	Starved
NMRI	57	300 ± 15	350 ± 10
CBA	58	310 ± 14	400 ± 32^1
Swisse	70	220 ± 24	400 ± 33^1
DBA	30	460 ± 43	430 ± 26
NMRI \times Swisse (obese)	51	280 ± 35	330 ± 33

It also shows the ratio of total body protein and total body fat indicating marked strain differences

Experiment 2

As shown by Table I the obese mice have a 15 per cent decrease in weight after 24 hrs without food. The carcass contents of protein and fat in the obese mice as well as the protein — fat ratio may be seen in Table II.

Table III gives the values of glucose and FFA in the blood

Experiment 3

Table III shows that the strains of mice respond differently to the starving period as judged by blood glucose and FFA values

Discussion

The present results indicate that the type of strain of mice plays an important role when studying nutritional problems. The response of the animals to a given diet with regard to carcass composition thus varies from strain to strain. It has previously been shown that strain differences in mice exist concerning fat mobilization, cardiac glycogen and other metabolic parameters (*cf* Fenton 1960). Certain types of obesity is known to be hereditary in mice (*cf* Mayer 1963) and in dogs (Mayer 1963 and Krook, Larsson and Rooney 1960). Two types of obesity have been recognized namely regulatory due to dysfunction of the hunger-satiety mechanism and metabolic due to a peripheral disorder in metabolism (*cf* Mayer 1963).

The present results indicate that on a given diet the strains of mice studied differed with regard to carcass composition (Table II). Thus, in the fed state the CBA strain deposited more fat than the other strains. When fasted for 24 hours, which in reality means a starvation in this species due to high basal metabolic rate, all strains decreased in weight. The amount of carcass fat that disappeared during the 24 hrs varied, however. The results indicate that the Swisse mice, in the fed state rather "slim", lost more fat relatively during these 24 hrs than did the CBA and NMRI strains. This suggests a higher fat mobilizing capacity of the Swisse mice as compared to the other strains studied. In the Swisse mice the epididymal fat pad disappeared almost entirely after 24 hrs of fasting. As may be seen in Table III the blood sugar at the same time went down to a hypoglycemic value. It was also found that 24 hrs without food was critical with regard to the IFA values of the blood. A fasting period longer than 24 hours significantly lowered the blood FFA in the Swisse mice whereas 16–24 hrs without food gave elevated values compared to those in the fed state.

In contrast to the Swisse mice the NMRI and CBA strains did not seem to mobilize fat so readily after starving for 24 hrs. This is supported by the finding that these strains showed less elevation of FFA when fasted and a relatively smaller weight loss. The obese mice behaved more similar to the NMRI and CBA strains than to the Swisse mice. The obese mice used were NMRI x Swisse to combine the two strains. The CBA and partly the NMRI mice can be regarded as fat animals. At least the CBA strain has been found to be very sensitive to variation of the protein value of the diet, with regard to fat deposition (Munck 1964). Thus, a diet with low biological value will produce obese animals not judged by the weight but by the carcass content of fat. The diet used in the present study had a high value of protein both quantitatively and qualitatively. Still high contents of carcass fat was found in the CBA and NMRI mice.

In many respects the DBA mice did not fit in with the results from the other strains. In spite of an appreciable decrease in fat content of the body when starved for 24 hrs, the FFA level of the blood rather decreased. The level of IFA in the fed state was

complex central integration mechanisms involved normally and eliminating the hazards of adaptation. This paper describes an attempt to perform such a study, where some of the results have earlier been briefly reported (Folkow and Rubinstein 1964).

Material and Methods

fixating them to the skull

Acute experiments. A group of 20 rats (Sprague Dawley strain) of both sexes ranging in weight from 200 to 300 g were used in acute experiments under nembutal anesthesia for the localization of the hypothalamic area which in the rat integrates the characteristic autonomic pattern of the defence reaction. The animals were placed in a stereotaxic instrument designed for using de Groot's stereotaxic atlas of the rat's brain. Arterial blood pressure was measured from a cannulated carotid artery with a Statham P23Db transducer. respiration was monitored from a side tube on the tracheal cannula with a Statham P23AC transducer. Renal, intestinal and muscle blood flows were measured by cannulation of the renal, mesenteric and femoral (skinned leg) veins respectively conducting the venous outflow through silicon oil filled lucite drop chambers and then returning the blood to the animal via the jugular vein. The drops were counted by a photoelectric cell activating an ordinate writer or one of the channels of a Grass Polygraph where all the above mentioned variables were recorded.

Chronic experiments. Another 40 rats were selected at random for the chronic experiments with repeated hypothalamic stimulations. A single stainless steel electrode (0.5 mm bare tip) was stereotactically implanted in each animal in the area which in the acute experiments induced the typical autonomic defence reaction. The occurrence of a blood pressure rise (tail method) and heart rate acceleration during stimulation was checked after the implantation while the rats were still asleep. After full recovery from the anesthesia the stimulation induced modifications in spontaneous behaviour were studied on the freely moving rats (attached to the stimulator by long lightweight

All the rats, both stimulated and controls, were placed in individual cages and their electrodes

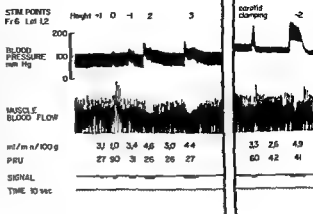
(1700 to 0500 hrs). The stimulus intensity was deliberately set below the level needed for evoking

stimulations protruded the tail relaxed he was coupled anducer in the experiments on anesthetized rats where the means of a Statham P23Db trans-

the distal cuff — In a nut sure was simultaneously

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Fig 1 Anesthetized rat Topical hypothalamic stimulation at frontal 6 lateral 12 and at different dorsoventral heights, coordinates according to de Groot's atlas As seen from the blood pressure and muscle blood flow recordings a blood pressure rise and a muscle blood flow increase are induced at heights -2 and -3 Atropine 0.3 mg/kg is given between the two panels — Stimulation at the same coordinates elicits in the awake animal increased alertness and ultimately an escape reaction



mean arterial blood pressure in the rat's tail; moreover, all the recordings were throughout performed by the same person and the procedure was standardized as far as was possible.

Each week mean blood pressure estimations were performed in every animal from both groups until three consecutive comparable readings were obtained for each rat. These three recordings from each animal were plotted together with the other data from the animals of the same group where ultimately only six entirely intact rats were left in each group. The mean and standard deviation of the

Wiley Ltd 1938)

Food and water intake of all the animals was measured daily for one week at regular intervals during the course of stimulation. All animals were given the same rat food and were kept in identical isolation cages throughout so that the intermittent hypothalamic stimulations in the stimulated group should constitute the only difference from the control group.

When the present series of experiments had to be interrupted after 4 months of repeated stimulations gross anatomical studies were conducted on heart, major arteries, kidneys, adrenals and gastrointestinal tract; the body weight and the organ weights were measured. The position of the electrode tips was checked with the usual procedure of serial 50 μ frozen sections of the brain fixed with 10% formaline.

Results

1. Acute experiments Topical stimulations were performed in anesthetized rats at 0.5 mm steps along the coordinates frontal 5 to 5.0 and lateral 0 to 1.0 corresponding to different locations in the hypothalamic area. Stimulation with 100 imp/sec, 1 msec duration and 1 to 3 V at coordinates frontal 6.0 lateral 1.2 and dorsoventral height -2.5 elicited hyperventilation and a characteristic pattern of sympathetic activation — tachycardia, acute hypertension, renal and intestinal vasoconstriction where blood flow decreased despite the raised perfusion pressure, and an increase

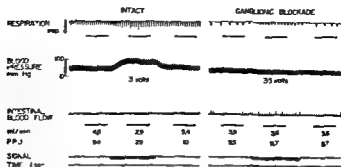


Fig 2 Anesthetized rat Recordings of respiration blood pressure and intestinal blood flow Topical hypothalamic stimulation at frontal the lateral 1.2 and dorsoventral height —2.5 before and after ganglion blockade with Arfonad (Roche) Note the increased respiration blood pressure rise and greatly increased intestinal blood flow resistance where the latter two changes are eliminated by Arfonad.

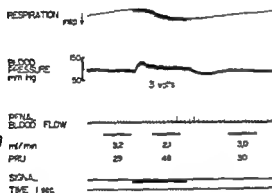


Fig 3 Anesthetized rat Effects on respiration blood pressure and renal blood flow of topical hypothalamic stimulation at the same site as in Fig 2 Note the considerable increase of renal blood flow resistance the blood pressure rise and the increased respiration.

muscle blood flow Fig 1 2 and 3 This response pattern is quite similar to the one observed e.g. in the cat during defence area stimulation (Folkow and Rubinstein 1963), except for the apparent absence of a cholinergically mediated active vasodilatation in the muscle vascular bed of the rat Thus atropine injection did not affect the vascular response in the skeletal muscles of the rat Other studies may, however, indicate that only some mammals mainly carnivorous animals like dogs, cats, etc., have a really significant supply of sympathetic cholinergic vasodilator fibres (Lvnas 1963)

II Chronic experiments With the electrodes placed at the coordinates mentioned above a group of animals was kept under a schedule of repeated hypothalamic stimulations as described in Methods The spontaneous behaviour of these animals when not actually exposed to a hypothalamic stimulation which always alerted them was apparently in other respects unchanged and similar to that of the control animals, and they kept their normal rhythm of activity

The main technical difficulty was to keep the wires and electrodes from breaking, and such events gradually reduced the number of intact animals in both groups

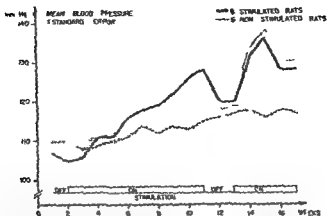


Fig 4 A diagram illustrating the gradual change of the resting mean blood pressure in 6 rats which for a prolonged period were intermittently stimulated in the hypothalamic defence area. As a

to be again rapidly raised when the stimulations are restarted

However a group of 6 stimulated animals and a corresponding number of controls were kept in excellent condition, tolerating well the isolation and restraint. The stimulations could here be maintained for about four months except for a period of "rest" of two weeks (between the 11th and 13th). The ultimate results will correspond then to those 6 rats that could be kept under the desired stimulation schedule, compared with the other 6 control animals living under exactly the same conditions, i.e. isolation, restraint, with one implanted electrode in the hypothalamus but no stimulation. The combined effect of the restrained living conditions plus the electric hypothalamic stimulations caused no significant modification in the food and water intake of the stimulated animals as compared with the controls. Also their body weight increased in a similar fashion as in the controls. These findings seem to exclude the possibility of gross accidental stimulations or electrode damages to e.g. the close by feeding center of the rat which is known to induce a significant increase in the food intake (Smith 1961).

The main findings are summarized in Fig 4 and Table I. In Fig 4 is depicted the course of the resting mean blood pressure during the weeks of intermittent hypothalamic stimulations in the group of 6 stimulated rats and in the control rats. As is seen from the figure the stimulated rats were throughout kept on the schedule of daily stimulations except for an interval between the 11th and the 13th week. The diagram shows a very moderate but gradual increase in resting blood pressure as recorded in the awake rats at least 6 hrs after the end of the period of stimulation of the day. The difference in the blood pressure levels started to be significant after the 8th week of daily stimulations. The interruption of the schedule for two weeks on the 11th and 13th week caused a significant decrease of the blood

TABLE I The table shows the mean blood pressure levels \pm standard error (S.E.) in the stimulated rats and the non-stimulated control rats during the entire period of experiment

Weeks	Stimulated group mean \pm S.E. 6 rats	Non stimulated group mean \pm S.E. 6 rats	Significance (p less than)
1	107 \pm 1.8	110 \pm 1.6	N.S.
2	105 \pm 1.9	110 \pm 2.0	N.S.
Stimulation on			
3	106 \pm 2.3	108 \pm 2.5	N.S.
4	111 \pm 2.2	109 \pm 1.9	N.S.
5	111 \pm 2.4	110 \pm 2.2	N.S.
6	116 \pm 2.3	111 \pm 2.2	N.S.
7	118 \pm 2.5	114 \pm 2.4	N.S.
8	119 \pm 2.4	112 \pm 2.3	N.S.
9	122 \pm 2.2	114 \pm 2.3	0.03
10	126 \pm 2.5	113 \pm 2.3	0.01
11	128 \pm 2.6	115 \pm 2.0	0.01
Stimulation interrupted			
12	120 \pm 2.1	116 \pm 2.2	N.S.
13	120 \pm 2.0	117 \pm 2.3	N.S.
Stimulation restarted			
14	131 \pm 2.4	118 \pm 2.0	0.01
15	136 \pm 2.3	116 \pm 2.1	0.01
16	128 \pm 2.2	118 \pm 2.2	0.02
17	128 \pm 2.1	117 \pm 1.9	0.02

pressure level in the stimulated rats, so that they approached the pressure levels of the controls. When the stimulation was resumed, the level of 'resting' blood pressure rose rapidly to the values seen in the stimulated rats just before the interruption, rather than showing the slow pressure increase that occurred when the stimulations were started. This new pressure rise was maintained at a significantly higher value as compared with the controls up to the end of the experiment.

The gross anatomical studies showed that the organs of the stimulated animals and their body weights were not significantly different from those of the controls. In only 2 of the 6 hypertensive rats there was a moderate but hardly significant increase in heart weight. No gross vascular changes were observed. It should here be stressed, however, that the stimulations had deliberately been kept fairly weak so as to create only a moderate intermittent impact on the cardiovascular system.

Discussion

The main requirement for this experimental approach was to find a hypothalamic area, from where it would be possible to elicit an autonomic activation corresponding to the defence reaction in other experimental animals and man, which evidently is normally activated in most alarm situations and in which

mental stress This was accomplished, as was mentioned in Results, by the series of acute experiments However, there was no evidence of a cholinergic vasodilatation in the muscle vascular bed of the rat As the engagement of these specific sympathetic fibres in the cat, at least, is considered an almost *sine qua non* component of the defence reaction (Abrahams, Hilton and Zbrozyna 1960), the lack of its finding in the rat may at first sight be somewhat surprising However, as mentioned earlier, it might be so that in mammals a really significant supply of cholinergic sympathetic vasodilator fibres to the skeletal muscles is present only in carnivorous animals (Uvnäs 1965) In all other respects the autonomic component of the defence reaction, and its somatomotor counterpart, was closely similar in the rat as compared with the cat

Since the experiments with chronic stimulations met with great technical difficulties they should, with respect to the results based on only the small number of animals ultimately left, be considered as preliminary ones, studies on a larger scale are needed In any case, this type of approach in animal experiments, linked to long term clinical studies in man (cf Charvat, Dell and Folkow 1964), both seem to be necessary for a reliable evaluation of the importance of mental stress for the gradual development of a chronic hypertensive state, as triggered by the centrally induced intermittent autonomic nervous and hormonal discharge which accompanies such situations

This small series of pilot experiments on chronic rats is promising insofar as the results suggest that a gradual shift of the resting blood pressure level really takes place in the stimulated animals This, despite the fact that the topical hypothalamic stimulations in the present experiments were throughout very mild and did not involve any drastic, acute disturbances of either behaviour or the cardiovascular equilibrium The gradually developed, significant shift in the resting blood pressure was, however, not sustained in the rats for long periods of time but approached the pressure level of the controls if the stimulations were interrupted for two weeks When, the stimulations started again, the resting pressure level was soon raised to the level seen just before the two week interruption, rather than showing the gradual rise, seen at the initiation of the stimulation period These findings are of interest for two reasons First, they render more confidence to the differences in pressure levels observed and make it far less likely that this difference was only due to chance Second they suggest that, in rats at least, the resting blood pressure equilibrium will tend to return towards normal once the trigger factor is eliminated at least as long as it is a matter only of a fairly mild degree of induced hypertension The gradual rise in resting blood pressure is certainly not a consequence of some sort of perpetuated hypothalamic after-discharge, induced by the brief periods of topical stimulations, because regularly the acutely induced cardiovascular adjustments disappear well within a minute after ended stimulation

Whether there is any direct correlation between the average peak increase in pressure during the intermittent stimulations and the rate and extent of the gradual increase of resting blood pressure level is not known at present How

being equal, it seems likely that the more the average blood pressure load is increased during a prolonged period, the more extensive secondary cardiovascular changes will become (cf Folkow 1960). It should, on the other hand, be realized that also other factors, besides the neurogenically induced load on the cardiovascular system, are involved in the defence reaction. For instance, topical activations of the defence area have been shown to trigger a profound release of ACTH, corticoids (Folkow, Hedner, Lisander and Rubinstein 1965), and also catechol amines, mainly adrenaline (Grant *et al* 1958). It is, in addition, likely that an increased aldosterone secretion is involved. This means that the impact of mental stress on the cardiovascular system should not only be considered as mediated via the autonomic nervous system *per se*, acting on the cardiovascular effectors directly. It will quite likely also involve in a most important way some more indirectly acting hormonal influences, which affect the cardiovascular system by changing aspects like the water-salt balance, thus affecting the blood volume, or the ionic equilibrium across the membranes of cardiovascular effectors, etc., which may considerably affect their 'reactivity' (Charvat, Deli and Folkow 1964). Thus, what may at first sight appear to constitute a purely neurogenic trigger factor, elicited from higher nervous centers in a way that mimicks the events taking place during mental stress, is far from purely neurogenic but rather should be considered as a complex of centrally elicited neurohormonal factors which in both direct and indirect ways affect the cardiovascular system.

It is not surprising that no gross morphological changes could be observed in the stimulated animals. After all, they had deliberately been exposed to only fairly weak excitations of the defence area and the gradual, secondary rise in resting blood pressure was so moderate that it can hardly be expected to have led to more obvious morphological changes in vessels or heart. It is possible that the situation would, in this respect, have been different in case more intense stimulations had been performed. This would, on the other hand, have greatly increased the risks of damaging the stimulation wires and electrodes. Further, other hypothalamic areas, controlling e.g. food and water intake, temperature regulation etc. might then also have been affected, causing gross disturbances of the animals' general homeostasis, a situation to be avoided in a project of this nature.

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AB Hassle Goteborg Sweden has generously covered the employment of a technician.

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Electrical stimulus

A Method for Intracardiac Recording of Monophasic Action Potentials in the Dog Heart in Situ

By

ULF SJOSTRAND

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Abstract

SJOSTRAND, U. *A method for intracardiac recording of monophasic action potentials in the dog heart in situ* Acta physiol scand 1966 68 58—63

and duration of the action potential

'An understanding of electrocardiographic complexes will be achieved when the potential at a given body surface point can be predicted from a knowledge of ventricular depolarization and repolarization pathways. To do this for QRS we must have exact information on three factors. First, we must know the time course and magnitude of potential changes across the membranes of the ventricular syncytium as depolarization takes place. Second, we must know the pathway of ventricular depolarization. Third, we must understand the basic principles of current flow in volume conductors and the modification of these principles necessitated by the resistive inhomogeneity of the tissues and the irregular shape of the body.

This citation is taken from a paper of Scher and Young published in the Annals of the New York Academy of Sciences 1957 and reflects what from a theoretical aspect is required for understanding of the electrocardiogram.

Regarded from a practical clinical aspect the main importance of electrocardiography lies in the fact that it gives in a relatively simple way essential information on the excitation, rhythmicity and impulse propagational conditions in the heart. By investigations on animals the appearance of the action potential in different regions of the heart has been studied *in vitro* and the impulse propagation has been studied *in vivo*. The information concerning excitation and propagation which is

obtainable from the potential changes recorded on the body surface, i.e. the electrocardiogram (ECG), applies to large and as a rule not absolutely definable sections of the conducting system and the atrial or ventricular muscle. For technical reasons (volume conductor recording) it is not possible with electrodes applied to the body surface to record the activity of the SA node or the electrical activity in other parts of the conducting system (e.g. the AV node or the bundle of His) for each individual cardiac cycle, analogically neither is it possible with external electrodes to record and localize the electrophysiological activity during the individual cardiac cycle in small anatomically defined regions of the atrial and ventricular muscle. Area Display Electrocardiography (Schuler and Roy 1965) appears, however, to give a spatial picture of the electrophysiological mean activity in relation to the ECG in relatively limited regions of the myocardium.

The size and course of the action potential in the human heart is only partially known. Only two studies of the transmembrane action potential in human atrium muscle (in vitro) have been published (Trautwein *et al.* 1962, Sleator and de Gubareff 1964), whereas the action potential in the different cardiac regions in both warm- and cold blooded animals has been relatively well investigated (for review see Weidmann 1956 a, Weidmann 1957). The path by which the impulse is propagated has been studied experimentally in the dog (for review, see Durrer *et al.* 1965, Scher 1965), in different types of monkeys (Scher 1965) and in man (Durrer *et al.* 1965). With regard to the distribution of the electrical activity of the heart out to the body surface, there is a good review by Gelernter, Swihart and Angell (1965).

A technique for intracardiac recording of action potentials in the intact mammal heart (including the human heart) should make it possible to study the subendocardially situated conducting system in both the atrium and the ventricle, and also to record the action potential in subendocardial atrial and ventricular muscle. The form and duration of the intracardially recorded monophasic action potential, and also its time relationship to the simultaneously recorded ECG, should provide information of the electrophysiological conditions at the recording point, thereby enabling a detailed analysis to be made in demarcated sections of the cardiac chambers. By roentgenological localization of the position of the recording point (recording of intracavitary ECG provides further possibilities for localization — especially as regards pacemaker regions) it should be possible with such a method to determine the normal conditions of impulse propagation both in man and lower animals and also the impulse formation and propagation in cases with a pathological ECG. Principal prerequisites for arrhythmia which have been established on the basis of experimental action potential studies in animals could by this means also be evaluated directly in the intact human heart. It should further be possible to study the effect of intracardiac pressure changes (Teorell 1965, drugs for review see Trautwein 1963), electrolytes and blood gas composition on impulse formation and impulse propagation in the heart. Furthermore by this method it should be possible during open cardiac surgery to localize conducting structures so as to avoid deleterious traumatic damage during the surgical procedure.

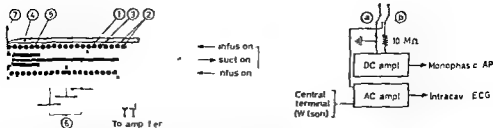


Fig 1a (left) The intracardiac end of the suction electrode equipment lying against the endocardial surface (semi schematic) 1 = polyethylene tube 2 = stainless steel wires one central and adjustable the other attached to the stainless steel tube at the tip 3 = stainless steel coil spring 4 = X ray opaque catheter 5 = stainless steel tube 6 = indication of adjustment possibilities relative to X ray opaque catheter 7 = endocardial surface By means of the switch shown in Fig 1b alternation can be made between electrogram — monophasic AP (position a) and intracavitary ECG (position b, AC-ampl — one of the alternating current amplifiers in the ECG writer) Fig 1b (right) Block diagram of the amplifiers and registration systems Cf legend of Fig 1a

Method

The intracardiac electrical activity (electrogram and monophasic action potential respectively) was recorded by means of a suction electrode technique (see e.g. Sjöstrand 1964). The suction electrode was of such dimensions and form that it could be passed through conventional heart catheters for clinical use. Fig 1a shows the part of the suction electrode equipment that was applied intracardially. The suction electrode consisted in this case of a polyethylene tube (Fig 1a 1) 800 mm long and with an inner diameter of 0.8 mm. A stainless steel wire (Fig 1a 2) with a diameter of 0.25 mm ran through the centre of the polyethylene tube. The polyethylene tube with the central stainless steel wire was then introduced into a coil spring of stainless steel wire (Fig 1a 3) diameter of wire 0.3 mm inner diameter of coil spring 1 mm. The stainless steel coil spring with its contents can be passed into an ordinary X-ray opaque plastic catheter (Fig 1a 4) Ödman-Ledin type grey, H&A Stockholm Sweden inner diameter 1.8 mm, intended for cardiac catheterization. In order to obtain better recording conditions a stainless steel tube (Fig 1a 5) was introduced into the end of the polyethylene tube and to this stainless steel tube was attached a 0.1 mm stainless steel wire (Fig 1a 2) which ran through the centre of the polyethylene catheter. As may be seen in Fig 1a the mutual positions of the central stainless steel wire the polyethylene tube the stainless steel coil spring and the X-ray opaque cardiac catheter could be regulated.

1964) conditions are created at the site of suction for good contact between the electrode and the protoplasm of the underlying cells (in order that any KCl leaking out shall not reach a high concentration outside the central suction catheter this is washed out with a solution with a normal NaCl concentration).

Sjöstrand 1966) recording is made with a Tektronix oscilloscope (type 361 Tektronix Inc. Portland, U.S.A.) and also parallel with this in one channel (direct current amplification) of a four channel Elema Mingograph (type 42 B Elema-Schonander AB Stockholm Sweden) in experiments on the dog the remaining three channels record the standard leads (subcutaneous electrodes). The frequency characteristics of the Mingograph do not permit a true recording of the form of the action potential, the recorder was accepted however since the time relation of the action potential was of interest. — As shown in Fig 1b intracavitary ECG can also be recorded in the usual way with the coil spring as the detecting and a central terminal (Wilson) is the other electrode.

The suction which is applied is about 600 mm Hg. The central suction electrode is filled with isotonic electrolyte solution in which part of the NaCl has been replaced with KCl so that the K concentration is 44 mM.

Experiments were performed both on toads (*Bufo bufo*) which had been decapitated and on dogs which had been anaesthetized with a combination of chloralose and Nembutal. In frogs the

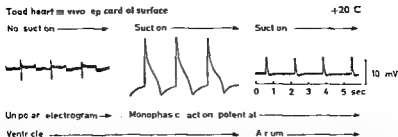
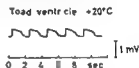


Fig 2 Three recordings from the same toad heart (in vivo) where the tip of the suction electrode equipment (cf Fig 1a) is placed against the epicardiac surface (Elema Mingograph 42 B)

Fig 3 Recording from toad heart (epicardiac surface ventricle) with the suction electrode equipment mentioned on p 5 (experiments on man)



suction electrode equipment was applied to the exposed epicardiac atrial or ventricular surface — these experiments were performed in order to ascertain by an inexpensive and easy method some of the principal characteristics of the recording electrode equipment. In the dog the equipment was introduced via the right femoral vein and the tip of the catheter was placed in position by direct intrathoracic manual means via a right sided intercostal (about 3 cm long) thoracotomy (for this the dog was ventilated at excess pressure)

Results

Fig 2 shows an experiment on the toad. On placing the electrode against the ventricular surface a ventricular electrogram is recorded, which on suction and slight penetration of the central stainless steel wire is transformed to a monophasic action potential. A similar but somewhat smaller action potential is also obtained, from the electrode when applied in the same way in the atrium. Fig 3 shows a recording from the ventricular surface in another toad, in which a better plateau was obtained (with regard to suction electrode recordings from the sinus venosus and atrium in the toad reference may be made to Sjostrand 1964).

Fig 4 shows ECG (standard leads) and action potential recordings in dogs. In this case (a) the suction electrode was applied in or in the vicinity of the sinus node region, and as may be seen in the figure the recorded action potential has a depolarization phase appearing before the P wave in the electrocardiogram on recording with the four channel writer (same time scale for all recordings). The figure also shows a recording (b) with the suction electrode applied in the apical part of the right ventricle, and it may be seen that the depolarization phase of the recorded action potential coincides with the QRS complex which is in complete agreement with what is known of the impulse propagation in the dog heart and what has also been accepted for the human heart (cf Goldman 1964). Fig 5 shows an oscilloscopic recording of the same section as Fig 4 (b).

Discussion

With regard to the relationship between the action potential recorded by the micro-electrode technique and that recorded by the suction electrode technique reference

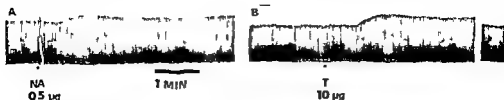


Fig. 1 Perfusion of isolated heart of *Lampetra fluviatilis*

A Effect of noradrenaline (NA) 0.5 µg

B Effect of tyramine (T) 10 µg

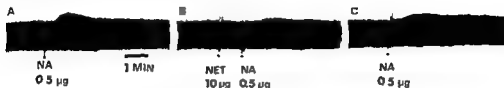


Fig. 2 Perfusion of isolated heart of *Lampetra fluviatilis*

A Effect of noradrenaline (NA) 0.5 µg

B Effect of noradrenaline (NA) 0.5 µg 1 min after injection of Nethalide (NET) 10 µg

C Effect of noradrenaline (NA) 0.5 µg 25 min after the injection of Nethalide 10 µg

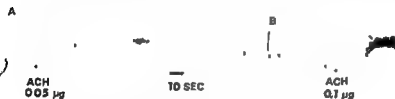


Fig. 3 Perfusion of isolated heart of *Lampetra fluviatilis*

A Effect of acetylcholine (ACH) 0.05 µg

B Effect of acetylcholine (ACH) 0.1 µg

of Augustinsson *et al* (1956). Isoprenaline also produced positive inotropic and chronotropic effects of the same nature as that of adrenaline and noradrenaline. The effect of the different agents was in the order isoprenaline > adrenaline > noradrenaline. Lowest effective dose of adrenaline was 0.05 µg. In hearts sensitive to adrenaline and noradrenaline, tyramine in doses of 10–25 µg caused positive inotropic and chronotropic effects similar to those of adrenaline and noradrenaline (Fig. 1).

The positive inotropic and chronotropic effects of adrenaline, noradrenaline and isoprenaline were readily blocked by Nethalide (Fig. 2), a β -receptor blocking agent (Black and Stephenson 1962).

Acetylcholine and nicotine were reported by Augustinsson *et al* (1956) to produce a negative inotropic and a positive chronotropic effect in the isolated lamprey heart. These authors also reported an inhibition of the effect of acetylcholine by curare. These observations were verified in the present investigation. In some hearts acetylcholine was effective in doses of 0.05–0.1 µg (Fig. 3) and in these more sensitive hearts 10 µg of nicotine produced an acetylcholine-like effect. The response

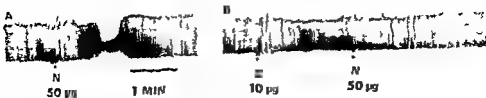


Fig. 4. Perfusion of isolated heart of *Lampetra fluviatilis*

A Effect of nicotine (N) 50 µg

B Effect of nicotine (N) 50 µg after pretreatment with curare (C) 10 µg

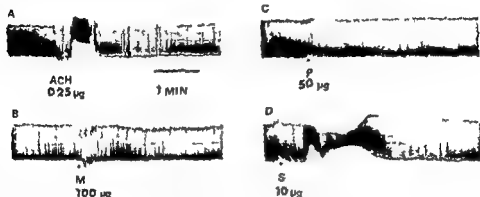


Fig. 5. Perfusion of isolated heart of *Lampetra fluviatilis*

A Effect of acetylcholine (ACH) 0.25 µg

B Effect of diisopropyl muscarine (M) 100 µg

C Effect of pilocarpine (P) 50 µg

D Effect of succinylcholine (S) 10 µg

to acetylcholine was not influenced by atropine nor was the effect of nicotine inhibited by hexamethonium but the effects of both acetylcholine and nicotine could, however, be blocked by curare (Fig. 4). Further succinylcholine in doses of 10 µg produced an effect similar to that of acetylcholine and nicotine (Fig. 5). The effect of succinylcholine was also inhibited by curare but not by atropine. di-Isopropyl muscarine and pilocarpine were tested in doses up to 100 µg without altering the rate and contractile force of the lamprey. Three out of the 20 hearts were insensitive to acetylcholine. One of these belonged to the group which was insensitive to the catecholamines and tyramine.

B. *Pleuronectes platessa*

Adrenaline, noradrenaline and isoprenaline produced positive inotropic and chronotropic effects on the isolated heart of the plaice. The effect of these agents was in the order isoprenaline > adrenaline > noradrenaline, isoprenaline being effective in doses of 0.05–0.1 µg. The responses to adrenaline, noradrenaline and isoprenaline were blocked by Nethalide.

Tyramine injected in doses up to and including 0.1 mg was ineffective. Higher doses were not tested.

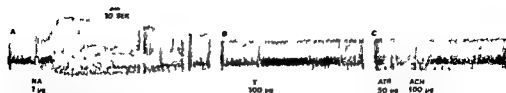


Fig 6 Perfusion of isolated heart of *Pleuronectes plateia*

A Effect of noradrenaline (NA) 1 μ g

B Effect of tyramine (T) 100 μ g

C Effect of acetylcholine (ACH) 100 μ g after pretreatment with atropine (ATR) 50 μ g

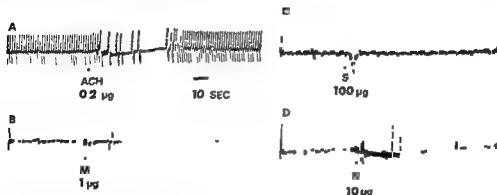


Fig 7 Perfusion of isolated heart of *Pleuronectes plateia*

A Effect of acetylcholine (ACH) 0.2 μ g

B Effect of di muscarine (M) 1 μ g

C Effect of succinylcholine (S) 100 μ g

D Effect of nicotine N 10 μ g

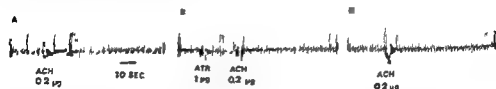


Fig 8 Perfusion of isolated heart of *Pleuronectes plateia*

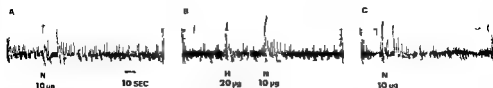
A Effect of acetylcholine (ACH) 0.2 μ g

B Effect of acetylcholine (ACH) 0.2 μ g after pretreatment with atropine (ATR) 1 μ g

C Effect of acetylcholine (ACH) 0.2 μ g 15 min after the injection of atropine 1 μ g

Acetylcholine in doses of 0.1–0.2 μ g and di muscarine in a dose of 0.5 μ g produced negative inotropic and chronotropic effects (Fig 7) which could be blocked by atropine (Fig 8). Succinylcholine in doses up to 0.1 mg was without effect (Fig 7). Nicotine in a dose of 10 μ g produced negative inotropic and chronotropic effects of the same nature as that of acetylcholine and muscarine (Fig 7), and the effect of nicotine was blocked by hexamethonium (Fig 9). When two injections of nicotine were given in rapid succession the heart became insensitive to this second dose. After 10–15 min rest the response to nicotine returned to normal again.

A survey of the different agents tested and their effects on the isolated hearts of the two species is given in Table I and II.

Fig 9 Perfusion of isolated heart of *Pleuronectes platessa*

- A Effect of nicotine (N) 10 μ g
 B Effect of nicotine (N) 10 μ g after pretreatment with hexamethonium (H) 20 μ g
 C Effect of nicotine (N) 10 μ g 15 min after the injection of hexamethonium 20 μ g

TABLE I Perfusion of isolated heart of *Lampetra fasciatus* Survey of the effect of different agonists and corresponding antagonists

Agent tested (Agonists)	Effect	Effect inhibited by (Antagonists)
Acetylcholine	Neg inotr and pos chronotr	d tubocurarine
di Muscarine	0	
Pilocarpine	0	
Succinylcholine	Neg inotr and pos chronotr	d tubocurarine
Nicotine	Neg inotr and pos chronotr	d tubocurarine
Adrenaline	Pos inotr and chronotr	Nethalide
Noradrenaline	Pos inotr and chronotr	Nethalide
Isoprenaline	Pos inotr and chronotr	Nethalide
Tyramine	Pos inotr and chronotr	Nethalide

TABLE II Perfusion of isolated heart of *Pleuronectes platessa* Survey of the effect of different agonists and corresponding antagonists

Agent tested (Agonists)	Effect	Effect inhibited by (Antagonists)
Acetylcholine	Neg inotr and chronotr	Atropine
di Muscarine	Neg inotr and chronotr	Atropine
Pilocarpine	Neg inotr and chronotr	Atropine
Succinylcholine	0	
Nicotine	Neg inotr and chronotr	Hexamethonium
Adrenaline	Pos inotr and chronotr	Nethalide
Noradrenaline	Pos inotr and chronotr	Nethalide
Isoprenaline	Pos inotr and chronotr	Nethalide
Tyramine	0	

Discussion

The variation in sensitivity of the lamprey hearts to the test agents is not understood. It could neither be correlated to sex or age or the period of time the animals had been stored before use nor to any histochemically detectable changes in the

catecholamine stores. Hearts which were sensitive to adrenaline and noradrenaline could be insensitive to acetylcholine and vice versa but most hearts, however, responded to adrenaline, noradrenaline and acetylcholine. Nevertheless there was one heart which was unresponsive to all these agents.

As stated previously, hearts of the lamprey, which were insensitive to noradrenaline and adrenaline were also insensitive to tyramine. Otherwise tyramine caused positive inotropic and chronotropic effects. On the other hand, hearts of the plaice were insensitive to tyramine, nor did large doses of acetylcholine produce any sympathomimetic effect after atropine.

It is well known that in organs where catecholamines have been depleted by reserpine treatment, tyramine is without effect but on treatment with noradrenaline or one of its precursors the effect of tyramine is restored (for references see Andén *et al.* 1964). While the effect of tyramine on the isolated lamprey heart may be explained by the release of existent stores of catecholamines (Dahl *et al.* 1966), its lack of effect on the plaice heart may be due to the absence of stores of that kind (von Mecklenburg 1966). Since no adrenergic fibres exist in any of these hearts the catecholamines released by tyramine in the lamprey heart must come from the catecholamine containing cells. Also, on the isolated hearts of both the lamprey and the plaice adrenaline, noradrenaline and isoprenaline produced positive inotropic and chronotropic effects which are readily blocked by a β receptor blocking agent (Nethalide). It would thus appear that the adrenergic receptors in these hearts are of the same nature as those in the mammalian heart i.e. of the β type according to Ahlquist's definition (Ahlquist 1948, 1962).

In the isolated mammalian heart nicotine produces a biphasic response, initially negative inotropic and chronotropic effects followed by positive inotropic and chronotropic effects. Both responses are blocked by ganglionic blocking agents (Kottogoda 1953). Only the initial response is blocked by atropine indicating that this part of the nicotine effect is due to the stimulation of cholinergic receptors. The second response of this drug is blocked by β receptor blocking agents and should thus represent the stimulation of adrenergic receptors. According to Burn and Rand (1958) the positive inotropic and chronotropic responses are due to the release of catecholamines from storage sites in the heart.

In the isolated lamprey and plaice hearts nicotine does not produce any biphasic response. In the former nicotine causes a positive chronotropic and a negative inotropic effect which are unaffected by hexamethonium. Acetylcholine and succinylcholine produce effects similar to that of nicotine which are not antagonized by atropine. The effects of all three nicotine, acetylcholine and succinylcholine are, however, blocked by curare. Since pilocarpine and muscarine are also ineffective in this preparation, the only existing cholinergic receptor in this heart seems to be of the curare sensitive type. After atropinisation of the mammalian heart larger doses of acetylcholine produce positive inotropic and chronotropic effects due to the release of catecholamines (for references see Burn and Rand 1965). In the lamprey heart, larger doses of acetylcholine do not produce any sympathomimetic effects.

after pretreatment with curare (Fig. 6). Thus the catecholamines in the heart of the lamprey are not released by nicotine or acetylcholine.

In the isolated plaice heart nicotine produces negative inotropic and chronotropic effects of the same nature as that of acetylcholine and which are readily blocked by hexamethonium and atropine. A negative inotropic and chronotropic effect is also produced by muscarine and the effect of this agent is likewise blocked by atropine.

The cholinergic receptors in the plaice heart thus appear to be similar to those in the mammalian heart in that they are of the hexamethonium and the atropine sensitive type.

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Some Properties of the Thalamic Relay Cells in the Spino-Cervico-Lemniscal Path

By

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Abstract

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Cells in nucleus ventralis posterolateralis thalami lateral to the relay cells of the hindlimb component of the dorsal column pathway have been studied with extra- and intracellular recordings in cats anesthetized with pentobarbital sodium. The cells in this part of thalamus are characterized by a

se
be antidromically invaded from the ipsilateral cortical areas S I and S II. A number of cells were antidromically invaded from both S I and S II indicating that these cells project to cortex with a divided axon. Many cells showed an inhibition of the recurrent type.

The thalamic relay of the spino-cervico lemniscal tract (SCLT) is located in a border zone laterally in the nucleus ventralis posterolateralis (VPL). In this region, convergence is observed between this path, the dorsal column lemniscal path (DC) from the hindlimb, and skin afferents from the forelimb (cf. Landgren, Nordwall and Wengström 1965). The response evoked from the SCLT has a shorter latency and is usually larger in amplitude than the response evoked from the dorsal column path (DC) or from the forelimb. In order to explain the great efficiency of excitation via the SCLT it was considered desirable to study the excitatory events in its thalamic relay station by intracellular recording.

The SCLT is known to have a cortical projection to S I and S II (Morin 1955, Norrsell and Voorhoeve 1962, Andersson 1962). Therefore, the cortical connections of the cells in the border zone were studied by antidromic activation from different cortical points. Such a study is of particular importance for cells with convergence from different peripheral sources since in the posterolateral part of the ventral nucleus

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(VPL) such cells have been assumed to be interneurons (Andersen, Eccles and Sears 1964 b)

In this report evidence will be presented indicating a thalamo-cortical projection from thalamic cells activated from SCLT only, as well as from cells receiving afferent excitation from several peripheral sources. It will also be shown that cells in this thalamic region have bifurcating axons projecting to two different cortical regions, located in Σ I and Σ II respectively. Evidence concerning inhibitory interaction between the afferent paths to the cells in the border zone will also be presented. These findings indicate that these cells are subject to a similar type of recurrent inhibition as demonstrated for neurones in the main part of the VPL by Andersen, Brooks, Eccles and Sears (1964 a)

Methods

overlying fimbria and hippocampus

The head of the animal was fixed in a Horsley Clarke holder and the microelectrode was inserted into the thalamus according to Horsley Clarke co-ordinates.

The brain was covered with a pool of paraffin oil at 37° C. The cortical potentials evoked by elec-

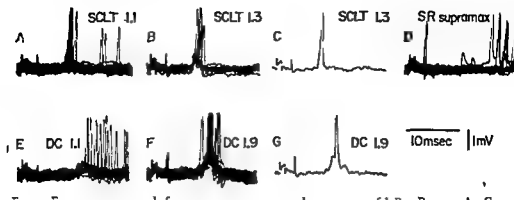
(Grass 4 S) and the stimulating current measured by a current probe (Hewlett Packard 460 A)

The contralateral superficial radial, median and ulnar nerves were dissected for electrical stimulation with bipolar electrodes. The spinal cord was exposed in the low thoracic region. The dorsal columns and the contralateral dorsolateral funiculus were cut and dissected for stimulation in an ascending direction by bipolar electrodes. The forelimb nerves and the spinal fascicles were covered by warm paraffin in pools made from the skin flaps.

The recording electrodes were glass micro-pipettes filled with solutions of either 4M NaCl, 3M KCl or 2M potassium citrate having resistances of 2—10 M Ω . The signals obtained from the microelectrode were fed into a cathode follower circuit which was able to neutralize the capacity between the electrode and ground. To unblock the microelectrode the neutralizing amplifier could be brought into oscillation. The cathode follower also delivered a square wave pulse that indicated the resistance of the microelectrode. The neural activity was displayed both on an oscilloscope via an AC coupled preamplifier and on a second oscilloscope with a DC coupled input in order to record the membrane potential.

The localization of the spino-cervico-lemniscal border zone in Σ PL

The spino-cervico-lemniscal border zone is located dorsally, laterally and ventrally to the somatotopically organized part of Σ PL where the dorsal column lemniscal path from the hindlimb evokes a focal potential of maximal amplitude (cf Landgren *et al.* 1965). In the beginning of each experiment a grid of exploring tracks was made using a low resistance NaCl filled microelectrode. The electrode was directed according to Horsley Clarke co-ordinates and the landmarks on the dorsal surface of the thalamus were noted. After completion of the grid the transversal area of Σ PL was known in terms of manipulator-co-ordinates. The microelectrode was then replaced by a high resistance microelectrode for recording from single neurones. The vertical co-ordinates of the new electrode were related to those of the exploring one by means of the haircross of an optical device in a fixed position on the stand carrying the micromanipulator. Additional information concerning the location of the recording sites was obtained during each subsequent track by observing the pattern of convergence between the different afferent inputs.



the resistance of the microelectrode

Results

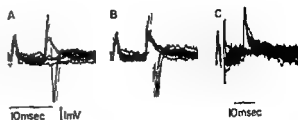
Pattern of convergence

Intracellular recording from cells in the spino cervico lemniscal border zone of *n. ventralis postero lateralis thalami* (VPL) is difficult because the cells in this region are small in size (mean diameter 19μ SD 5μ). Nevertheless, it was possible to investigate a number of neurones intracellularly under reasonably stable conditions and with resting potentials in the order of 20–60 mV. Additional information was obtained from records of extracellular giant spikes. Altogether 52 cells in the zone of convergence were investigated. 21 cells were excited only from SCLT and the remaining 31 cells were in addition excited from DC and/or forelimb nerves.

Fig. 1 shows extracellular records from a border zone cell that was excited by electrical stimulation of the SCLT and of the DC at the level of L1, and of the superficial radial nerve (SR). At a stimulus strength of 1.1 times threshold of the thalamic response (T) the SCLT gave an excitatory postsynaptic potential (EPSP) in most of the trials. The cell discharged on the summit of the EPSP with the exception of a few action potentials which appeared with a latency that was about twice as long (Fig. 1A). When the stimulus strength was increased to 1.3 T (B) the EPSP appeared in every sweep and the action potentials all had a rather constant latency.

Stimulation of the DC at a strength of 1.1 T (E) gave an EPSP with a longer latency. The action potentials appeared asynchronously on the falling phase of the extracellularly recorded EPSP. With a stimulus strength of 1.9 T (F) the action potential appeared earlier on the EPSP and the response was divided into two peaks. The duration of the EPSP evoked from the SCLT was much shorter than that produced by the DC, as can be seen by comparing records A and F. The single records in C and G show the difference in the rising phase of the EPSP evoked from stimulation of the SCLT and the DC. The SCLT gave a steep rising phase and the action potential was elicited on the crest with very little delay. The DC gave a slower

Fig 2 Intracellular records from a cell excited only from SCLT. Record A obtained at threshold strength and B at a slightly higher intensity of the stimulus show the unitary character of the synaptic action of the SCLT volley. In record C the threshold strength stimulus to the SCLT was preceded by an electrical stimulus to the cortical area S II giving antidromic invasion of the cell. Membrane potential 30 mV.



and irregular rising phase of the EPSP and the action potential appeared on the falling phase with a considerable delay. The small hump that appears after the action potential in record G probably corresponds to the second group of action potentials in record F. The cell was also discharged by stimulation of SR but with a longer latency (D) and sometimes with multiple spikes. The EPSP is obviously polysynaptically produced. The unitary EPSP with shorter latency that appears occasionally indicates involvement of polysynaptic chains with a relatively low safety factor of propagation.

The pattern of excitatory convergence illustrated in Fig. 1 was typical of many cells in the border zone. Other cells were discharged from the spinal fascicles only, or from one of the spinal fascicles and from one or several forelimb nerves. Neurones were also found that received excitation only from one source, either the SCLT, the DC or one of the forelimb nerves, although focal potentials and activation of cells close by were obtained following stimulation of the SCLT, DC and forelimb nerves, which indicated that these cells were located in the border zone and not in the somatotopically organized part of the nucleus.

When the synaptic action of a stimulus to the SCLT was studied intracellularly, it was repeatedly observed that the EPSP appeared with a constant size or not at all at stimulation intensities close to the threshold. Fig. 2 illustrates unitary EPSPs from a cell excited from the SCLT only. With stimulus strength at the threshold value of the thalamic response (A) it gave either no response, an EPSP of a constant amplitude or a somewhat larger EPSP that fired the neurone. At a slightly higher stimulus intensity the same two EPSP levels were seen (B). The all or nothing character of the EPSPs indicates that these are unitary, i.e. due to the excitatory synaptic action of the terminals of one single afferent fibre. The records in Fig. 3 show that in this cell the summation of only two unitary EPSPs was required in order to discharge a propagated action potential. Therefore excitation of only two neurones in the SCLT seems necessary for a propagated discharge. Since there was not visible break in the summated EPSP the fibres exciting the cell apparently had nearly the same conduction velocity. The fibres in the SCLT seem to constitute a rather homogeneous group as is also evident from the small rise in the stimulus intensity necessary to obtain a maximal response as compared with the threshold response.

(Landgren *et al* 1965) The constancy of the latency and amplitude of the excitatory action from the SCLT is further illustrated in Fig. 2, C. Here the cell was also antidromically discharged from the cortex (S II). A following stimulus to SCLT gives a unitary EPSP or no response at all.

Although unitary EPSPs were most commonly obtained in response to stimulation of the SCLT, such EPSPs were also observed in response to stimulation of the forelimb nerves or the DC. One cell, excited from the DC only, seemed to be fired by one unitary EPSP. When the cell was studied intracellularly, the action potential was found to be fired on the crest of the EPSP, or there was no response at all. It should, however, be noted that stimulation of the DC or the forelimb nerves in most cells gave an EPSP that grew continuously as the stimulus strength was increased above the threshold value until it reached the firing level.

In summary, our findings indicate that there are cells in the border zone which can give a propagated discharge by the excitatory action of one or two afferent fibres, most often belonging to the SCLT.

Cortical connections

In order to investigate the cortical connections of the cells in the border zone, brief electrical shocks were applied to the somatosensory areas S I and S II as well as to the surrounding cortex by means of a set of bipolar electrodes, the poles of which were equally spaced. The effects on the thalamic cells were analyzed with regard to monosynaptic and antidromic activation. The criteria used for this classification were: latency of discharge, the configuration of the spike and the ability to follow high frequency stimulation.

It was often observed that a cell in the border zone could be excited from two distinctly different cortical points. Such a cell is illustrated in Fig. 3. This cell was excited from SCLT (latency 5.9 msec) and from DC (latency 6.6 msec). The cell was tested for excitatory effects from the cortical points shown by the drawing in Fig. 3, and with the current intensities in mA as indicated for each point. The cell could be discharged only from the three points indicated with filled circles. The records obtained in response to stimulation of these points are shown in Fig. 3, A—C. The cell was fired with the lowest stimulus intensity (1.2 mA) from point A in the medial part of S I. The latency was 3.2 msec (Fig. 3 A). When the stimulus was applied to point B laterally and caudally to point A, the cell discharged irregularly and only if the stimulus intensity was increased to 16.5 mA, i.e. 14 times, but the latency was not significantly changed (Fig. 3 B). The cell was also discharged from an area in S II (cortical point C), separated from the focus in S I by an inexcitable zone. The stimulus strength required in S II was 5 mA and the latency of the spike was 1.4 msec. The antidromic nature of the response is suggested by the configuration of the spike which rises from the baseline without any visible prepotential. There is, however, a high break in the rising phase of the spikes due to change in risetime between the initial segment (IS) and the soma-dendritic (SD) spikes. A soma-dendritic blockage is seen in Fig. 3 D.

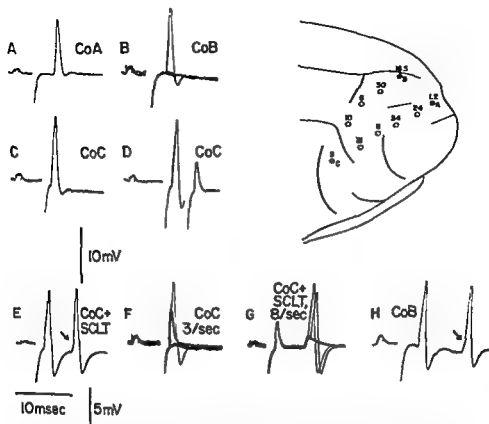


Fig 3 Intracellular records from a cell antidromically invaded from the cortical areas I and S 11. The diagram to the right shows the location of the stimulated points and the stimulus strength in mV. No short latency responses were obtained from points indicated with open circles. Records A, B, and C were obtained from the points marked A, B, and C (filled circles) with thresholds as indicated. Record D shows SD blockage to the second stimulus when paired shocks (interval 4 msec) were applied to point C. Record E shows simultaneous stimulation of the SCLT and the cortical point C.

The difference in the configuration between antidromic and transynaptic activation of this cell is illustrated in Fig 3 E—H. In E the SCLT and the cortical point C were stimulated simultaneously with a stimulation rate of 1 per second. Again, the first spike, elicited by the cortical stimulation, arose from the baseline without any prepontential and there was a pronounced break between the IS and SD spikes of the rising phase. The second spike, evoked by stimulation of the SCLT, was postsynaptic. It arose from a prepontential, indicated with an arrow, and had a very small break on its rising phase. In Fig 3 F are records showing stimulation at

old intensity of the cortical point C at a repetition rate of 3 per second. In one out of 10 trials a full spike was elicited, in the other the spike showed either a SD blockage or there was no spike discharges at all and no signs of an EPSP. In Fig. 3 G the cortical point C was stimulated at higher intensity together with the SCLT and at a repetition rate of 8 per second. In every sweep the cortical stimulation gave an IS spike. The SCLT response consisted of an EPSP upon which the action potential occasionally appeared. When the cortical point II was stimulated with high intensity, the cell sometimes discharged two action potentials (Fig. 3 H). The first spike arose from the baseline without any prepotential but with a distinct break in the rising phase. The later spike had a prepotential indicated with an arrow, of similar amplitude as the EPSP elicited from the SCLT but no clear IS—SD break. Although the latency of the spike response elicited from the points A and B in S I was long the records in Fig. 3 strongly indicate that this cell was fired antidromically from these points as well as from point C in S II.

The cell was also tested for its ability to follow high frequencies given as double pulses with different intervals. This particular cell followed paired stimulation of point C with an interval of 4 msec between the pulses but the SD component of the response to the second stimulus was then blocked (Fig. 3 D). An ability to follow cortical stimulation at frequencies as high as 600–800 per second was observed in some antidromically activated cells. Others were however blocked at frequencies above 10 per second. An inability to follow high frequency stimulation is to be expected because of the efficient and longlasting inhibitory action induced in thalamic cells by cortical stimulation (Andersen *et al.* 1964 a). Since some cells followed transsynaptic activation at frequencies of several hundred stimuli per second frequency following is not a good test for the differentiation between antidromic and transsynaptic activations.

Throughout the experiments 27 out of 39 systematically tested cells were invaded antidromically from the somatosensory areas. 14 of these cells were invaded from both S I and S II, 11 cells only from S I and 5 cells only from S II. Thus the results of our experiments have shown that a large number of cells in the spino-cervicoluminal border zone are discharged from two distinct foci in the hindlimb areas of S I and S II at a low stimulus intensity while other cells are antidromically invaded from only one of these areas. When the cells were discharged from both S I and S II the foci were separated by a cortical region stimulation of which caused the thalamic cells to respond only occasionally when very high stimulus intensities were used. The threshold for an antidromic activation of the SCLT cells was generally 2–3 mA although in several cells values below 1 mA were effective. The shortest latency was usually found when S II was stimulated. In many cells the threshold for the transsynaptic activation was lower than that of the antidromic response.

Inhibition

The somatosensory relay cells in the main part of the ventro-basal complex of the thalamus are subject to a powerful recurrent inhibition (Andersen *et al.* 1964 a).

Fig 4 Plotted curves showing the degree and time course of inhibition of the test response when preceded by a conditioning stimulus to SCLT, DC or the hindlimb area of S I. The cell was excited by SCLT and DC but not from cortex

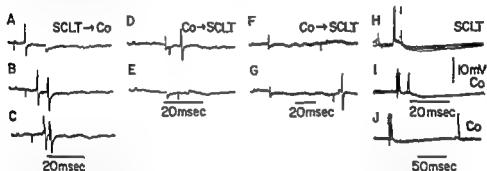
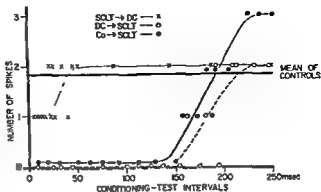


Fig 5 Extra (A—G) and intracellular (H—J) records of a cell showing excitatory as well as inhibitory actions from SCLT and the hindlimb area of S I. In records A—C a stimulus to SCLT precedes the cortical stimulation; in records D—G the conditioning stimulus is given to cortex. Record H shows the intracellular response to a SCLT volley; records I and J the response to stimulation of S I. Membrane potential 50 mV.

The cells in the border zone seem to constitute a less homogeneous group with regard to inhibition. Many cells with excitatory convergence or activated from only one peripheral source showed the same type of recurrent inhibition as the cells in the main part of the nucleus in being inhibited for 100–200 msec after stimulation of the afferent pathways as well as of the somatosensory cortex. In other cells, no signs of inhibition were found, either from the cortex or from peripheral sources. The most common pattern of inhibition was, however, that the inhibition elicited by stimulation of the SCLT was much weaker than that produced by the DC, the forelimb nerves or the cortex. The plotted curves in Fig 4 illustrate the inhibition observed in a cell which was excited by the SCLT and the DC pathway. The inhibition is given as a reduction in the number of spikes discharged by the test volley. When the SCLT volley preceded the stimulus to the DC the inhibition was weak and lasted less than 50 msec. A conditioning stimulus to DC however inhibited the response from SCLT completely for 150 msec. Although stimulation of the cortex did not discharge this cell, a conditioning stimulus to the hindlimb area of S I also inhibited the response from SCLT for about 150 msec. The recovery from the in-

hibition after a conditioning stimulus to the cortex was followed by a period of hyperexcitability with increased test discharges. Thus hyperexcitability appeared in many cells as a late discharge to the conditioning stimulus and with a latency of 100–200 msec (Fig. 5J) (cf. Andersen *et al.* 1964a).

Sometimes a more complicated interaction between the cortical effect and the effect of a peripheral stimulus was observed. Fig. 5 illustrates a cell activated by the SCLT and cortex. The cell was first studied extracellularly (records A–G) and later intracellularly (records H–J). During the extracellular recordings the cell was invaded antidromically from the hindlimb area of Σ I only when preceded by a stimulus to the SCLT at certain intervals (Fig. 5A–C). The facilitation of the antidromic response was due to an afterdepolarization following the discharge of the action potential by SCLT and preceding the hyperpolarization (H). When the cortical stimulation precedes the stimulus to SCLT a similar phenomenon was observed and there was a delay in the onset of inhibition of the SCLT response (D). As seen in the intracellular records (I and J) the cortical stimulus gave first an antidromic spike followed by a later transsynaptic action which occasionally discharged a second action potential. Thus, there is evidence of a short lasting mutual facilitation from the periphery and the cortex on relay cells in the border zone. This facilitation was followed by a hyperpolarization which was short lasting and weak when induced by the SCLT (H). The hyperpolarization elicited from cortex was more prominent and inhibited the SCLT response for 50 msec (Fig. 5D–G). The duration of the inhibition of the SCLT response was much shorter than the duration of the hyperpolarization (J) and the SCLT response is depressed only during the period of maximum hyperpolarization. This is an indication of the strong synaptic linkage in the excitatory path which can break through a moderate degree of inhibition. Fig. 5J also shows that the cortical stimulus induces a post inhibitory rebound excitation of a border zone cell.

Discussion

The present investigation is concerned with properties of cells in a part of VPL lateral to the region which receives a somatotopically organized projection from the dorsal columns. The cells are characterized by a considerable convergence with regard to their excitation by stimulation of afferent pathways. Thus, cells are activated from the contralateral spino-cervico-lemniscal and dorsal column pathways as well as from contralateral forelimb nerves either by one of these or by two or several in different combinations (Landgren *et al.* 1965). In the present experiments only cells excited by stimulation of SCLT alone or in combination with other peripheral inputs have been investigated.

Many thalamic cells excited by the SCLT were also excited from restricted foci in the cortical areas Σ I and Σ II at low stimulus intensities. The short latency action potential arose from the baseline without any prepotential in an all or nothing fashion and often showed a clear IS–SD break. These are characteristics of antidromic invasion of the cells. Furthermore, the response elicited by cortical stimuli

tion was compared with the response evoked transsynaptically by peripheral stimulation. The latter gave an action potential with a prepotential or only an EPSP at threshold intensities of the stimulus. Such a comparison supports the assumption of an antidromic invasion of cells in the lateral VPL following cortical stimulation.

The cortical response evoked by stimulation of the contralateral dorsolateral funiculus is mediated by the pathway of Morin (1955, *cf.* Andersson 1962). The first neurone of this pathway relays in the dorsal horn of the spinal cord of the same side from which it enters the cord (Lundberg 1964), the second neurone ascends in the lateral funiculus and relays in the lateral cervical nucleus. The third order neurone crosses the midline at C_1 – C_2 and ascends in the medial lemniscus to the VPL of the thalamus (Morin and Thomas 1955, Busch 1961). The present results show that the thalamic relay of this pathway is located in the border zone in the lateral part of VPL. Although some fibres ascending in the dorsal column medial lemniscus also terminate in this region, the bulk of these fibres terminate somewhat more medially in VPL. So far, cells activated from the hindlimb component of the SCLT and antidromically invaded from cortex have been found only in this border zone of VPL.

According to the criteria given above, a number of cells in the lateral part of VPL were excited antidromically by stimulation at low intensities of restricted foci in both S I and S II. This can be explained on the assumption that the thalamic cells project upon the cortex with a divided axon, one branch reaching S I and the other S II. Since the distance from VPL to S I and S II is approximately the same, the difference in latency between the invasion from the two cortical areas suggests that the branches have different conduction velocities and thus different diameters. Stimulation of S II usually gave an invasion of the cell with shorter latency than did stimulation of S I. Therefore it seems possible that some thalamic cells project to S II with their main axon while an axon collateral projects to S I. Evidence for divided axons was also found by Andersson, Landgren and Wolsk (1966) in their study of the cortical connections of the thalamic cells relaying group I input from the forelimb and in *nucleus ventralis posteromedialis*. Darian Smith (1964) found some cells which could be antidromically invaded from the face areas of both S I and S II.

Rose and Woolsey (1958) studied the degenerations in thalamus after cortical ablations. They frequently observed small focal degenerations in the posterior part of the ventro basal complex when S II was removed. If in addition the auditory cortex was ablated, the degenerative changes in this part of thalamus were most severe, although ablation of the auditory cortex itself only gave minor changes. The possibility exists that this type of facultative degeneration named sustained degeneration by Rose and Woolsey is due to a cortical projection with divided axons.

The cortical response evoked by impulses in the SCLT arising from the lower part of the spinal cord has a latency about 3 msec shorter than the response elicited by impulses in the DC pathway. Mark and Steiner 1958, Norrsell 1961.

1962, Andersson 1962) This latency difference may partially be due to a difference in the synaptic transmission. The efficiency of a synapse does not only depend on the magnitude of the depolarization produced by the incoming volley, but also on the rate at which depolarization occurs (Sasaki and Otani 1961). The recordings from the cells activated by the SCLT showed that this pathway gave an EPSP with a much steeper rising phase than the EPSP elicited by the DC pathway. Thus, the SCLT usually creates an EPSP that produces less accommodation of the membrane than does the DC pathway. The postsynaptic depolarization obtained from SCLT often occurred in unitary steps which most likely are due to the release of transmitter substance from a single SCLT axon. Summation of two or three unitary EPSPs elicited an action potential arising from the crest of the EPSP. Similarly large unitary EPSPs have also been observed in the lateral geniculate nucleus (Bishop, Burke and Davies 1962). No obvious time dispersion was found between the individual EPSP units. This is probably due to similar conduction velocities in the different SCLT fibres (Landgren *et al.* 1965). The amplitude of the EPSP obtained from the DC or the forelimb nerves usually grew gradually with increasing stimulus strength, and its rate of rise was slower than the SCLT-induced EPSP. In these circumstances accommodation of the membrane becomes an important factor, the safety factor of the synaptic transmission decreases with a concomitant increased synaptic delay.

The longlasting postexcitatory inhibition with repetitive afterdischarges of thalamic cells described by Andersen *et al.* (1964a) was also found in the lateral border zone of VPL. However, the inhibitory action elicited from the SCLT was usually weaker than that obtained from the DC pathway or from the forelimb nerves. This difference was particularly obvious in cells with excitatory convergence. Cortical stimulation elicited a strong inhibitory action of short latency in most of the cells in the border zone suggesting that the inhibition is effected by recurrent hyperpolarization. Since a large number of thalamic cells are invaded by an antidromic volley, the cortical stimulation is expected to give strong recurrent inhibition. The difference between the inhibitory action obtained from different peripheral sources may be due to differences in their excitatory connections. Thus, if the SCLT axons excite only a few thalamo-cortical neurones compared with those excited by the DC pathway only a small number of recurrent collaterals will excite interneurons with inhibitory action on the thalamic relay cells. Consequently the inhibition will be more restricted than would be the case if the afferent volley excited a large number of thalamo-cortical fibres (cf Andersen *et al.* 1964a). An alternative explanation may be sought in a different number of inhibitory interneurons in various parts of the VPL, and also in a varying degree of their axonal ramifications.

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Studies on the Mechanism for the Calorigenic Effect of Adrenaline in Man

By

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Abstract

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the calorigenic effect such as the heart stimulating and hyperglycemic effects of adrenaline is also considered

The calorigenic effect of catecholamines (CA) in animals and man have long been studied (reviews Lundholm 1949, Griffith 1951, Ellis 1956, Lundholm and Mohme-Lundholm 1960, Lundholm, Mohme-Lundholm and Svedmyr 1966). These studies indicate that the calorigenic effect of CA is probably not ascribable to one specific mechanism but constitutes the sum of several different effects, the relative importance of which may vary with the experimental conditions, animal species and CA derivative.

The way in which adrenaline (A) stimulates the oxygen consumption in man is of interest. Notable among the mechanisms which *a priori* may be of importance for this action are the glycogenolytic and lipolytic effects of A.

In experiments on the rabbit, Lundholm (1949) found that increased lactic acid production and metabolism could to a large part explain the calorigenic effect of adrenaline in this species. In later experiments in man Bearn, Billing and Sherlock (1951) found that the blood lactate concentration and the oxygen consumption increased during adrenaline infusion. The oxygen consumption of the liver was doubled, while at the same time its extraction of lactic acid increased. Noradrenaline had a weaker effect on the liver oxygen consumption.

Cobbold and Ginsburg (1960) found, however, that both noradrenaline (NA) and isoprenaline stimulated the oxygen consumption in man without increasing the blood lactate concentration appreciably. They therefore doubted the significance of increased lactic acid metabolism for the CA calorogenic effect.

Steinberg (1963), Steinberg *et al.* (1964) demonstrated a parallelism between the calorogenic effect of NA and its capacity to increase the plasma free fatty acid (FFA) concentration, and also showed that NA increased the oxidation of C^{14} labelled palmitic acid. Pronethalol blocked both the calorogenic and the FFA-mobilizing effect. They suggested that the calorogenic effect of NA was induced by increased metabolism of FFA.

Nicotinic acid (nic ac) has been shown to inhibit the FFA-mobilizing effect of CA but not its circulatory actions (Carlson and Oro 1962). Havel *et al.* (1964) selectively blocked the FFA-increasing effect of NA with nicotinic acid and found that the calorogenic effect of NA was thereby approximately halved.

The experiments described indicate that both increased lactic acid metabolism and FFA metabolism may be of importance for the calorogenic effect of CA in man.

In connection with studies on the influence of thyroid hormones on the calorogenic effect of adrenaline in rabbit and man (Svedmyr 1966 a, b, c, d) it seemed of interest to determine the relative importance of the mechanisms discussed above for the calorogenic effect of A in man.

In the present study, therefore, attempt was made to determine how great a role an increase in lactic acid metabolism and in FFA metabolism, respectively, played in the calorogenic effect of A in man. The contribution of the lactic acid metabolism was calculated by means of a regression equation which correlated the increase in oxygen consumption and increase in blood lactate concentration. These values were obtained on infusion of L(+)lactate in a quantity which gave about the same increase in blood lactate concentration as during A infusion. The FFA increase could not be reproduced by FFA infusion since free fatty acids are too toxic to administer i.v. The FFA mobilizing effect of A was therefore selectively blocked with nicotinic acid and the consequent reduction in the calorogenic effect of A was determined.

Method

The experiments were performed on a total of 6 healthy subjects of ages 22–30 years. Studies were made of basal metabolism and of the effect of A and of nic ac.

The subjects were fasted overnight and rested in a temperature controlled room (24.0 \pm 0.5 °C). A plastic catheter was inserted into a cubital vein in each arm. One of the catheters was used for the withdrawal of blood samples, via a three-way tap, and infusion of nic ac, and the other for infusion of A or lactate. When the catheters were not being used for these purposes 0.9% NaCl solution without addition of heparin was infused into each of them. After insertion of the catheters and confirmation of NaCl solution the subject rested for 60 min, after which the basal metabolism was measured during two 15 min periods. During each of these periods an aliquot of the basal samples were taken as then changed in the first series to A which was solved in 0.9% NaCl solution to which 0.1% of the metabolite was subsequently determined. The A infusion was subsequently determined by 85.9 and 11.1% in mm after the start of the A infusion. Blood samples were taken for analysis of blood lactate, blood glucose and plasma FFA.

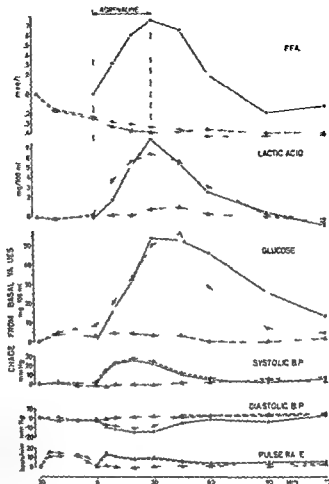


Fig. 1. Influence of A (●---●) on FFA, LACTIC ACID (Δ---Δ) and GLUCOSE (○---○) on some metabolic and regulatory functions in man. For further information see Fig. 1.

continued to increase reaching its maximum at the end of the infusion. It then decreased and regained the basal value 60 min after the end of the infusion. The CO_2 production did not run parallel with the O_2 consumption but reached its maximum as quickly as after 5 min decreasing then relatively more rapidly than the O_2 consumption. The ventilation increase ran parallel with the increase in CO_2 production. The pulse frequency increased 10–15 beats/min, the systolic blood pressure rose and the diastolic blood pressure fell during the infusion (Fig. 2).

The plasma FFA concentration rose continuously during the infusion, the maximal increase being 750 $\mu\text{eq/l}$. The blood lactate concentration rose almost linearly with the infusion time and showed a maximal increase of 8 mg at the end of the infusion. 60 min later it regained the basal level. The blood glucose concentration showed a maximal increase of 54 mg at the end of the infusion and then decreased slowly, but the basal level was still not

TABLE I The influence of adrenaline (Adr) nicotinic acid (Nic ac) and Nic ac and Adr in combination on O_2 consumption, CO_2 elimination and respiratory minute volume. Mean values \pm S.E. in 5 fasted subjects. Adr 0.10 μ g/kg/min infused for 30 min. Nic ac 5 mg/min infused for 150 min. Time in relation to the start of Adr infusion. P=probability that the effect was due to chance.

	Adr (I)	Adr+Nic ac (II)	Nic ac (III)	Mean difference in Adr effects with and without Nic ac I (II-III)
O_2 ml/kg/min				
Basal values	3.55 \pm 0.0823	3.47 \pm 0.084	3.41 \pm 0.128	
Change from basal values after -10-0 min				
0-10	0.48 \pm 0.039 P < 0.001	0.01 \pm 0.020 P < 0.005	0.00 \pm 0.066 -0.02 \pm 0.040	0.11 \pm 0.075
25-35	0.75 \pm 0.072 P < 0.001	0.47 \pm 0.065 P < 0.005	0.00 \pm 0.041	0.29 \pm 0.126
43-48	0.48 \pm 0.084 P < 0.005	0.30 \pm 0.051 P < 0.005	-0.06 \pm 0.080	0.11 \pm 0.072
55-65	0.25 \pm 0.114	0.14 \pm 0.066	-0.03 \pm 0.030	0.09 \pm 0.107
85-95	0.00 \pm 0.098	0.04 \pm 0.052	-0.02 \pm 0.036	-0.06 \pm 0.121
CO_2 ml/kg/min				
Basal values	2.76 \pm 0.083	2.73 \pm 0.087	2.72 \pm 0.151	
Change from basal values after -10-0 min				
0-10	0.83 \pm 0.039 P 0.001	0.02 \pm 0.044 P 0.005	-0.03 \pm 0.069 0.00 \pm 0.014	-0.08 \pm 0.078
25-35	0.63 \pm 0.061 P 0.001	0.78 \pm 0.086 P 0.001	0.11 \pm 0.053	-0.04 \pm 0.121
43-48	0.09 \pm 0.049	0.09 \pm 0.037	0.04 \pm 0.025	0.05 \pm 0.059
55-65	0.06 \pm 0.049	0.11 \pm 0.085	0.05 \pm 0.041	-0.01 \pm 0.081
85-95	0.05 \pm 0.036	0.16 \pm 0.051 P 0.05	0.21 \pm 0.050 P 0.02	0.10 \pm 0.072
Respiration volume l/min				
Basal values	5.77 \pm 0.167	5.55 \pm 0.197	5.64 \pm 0.244	
Change from basal values after -10-0 min				
0-10	1.80 \pm 0.111 P < 0.001	0.51 \pm 0.118 P 0.01	0.11 \pm 0.120	-0.20 \pm 0.268
25-35	1.46 \pm 0.185 P < 0.005	2.24 \pm 0.309 P 0.005	0.24 \pm 0.140	0.16 \pm 0.108
43-48	0.28 \pm 0.124	2.13 \pm 0.279 P 0.005	0.51 \pm 0.107 P 0.01	-0.13 \pm 0.157
55-65	0.29 \pm 0.124	0.86 \pm 0.099 P 0.001	0.44 \pm 0.082 P 0.01	-0.01 \pm 0.159
85-95	0.38 \pm 0.120 P < 0.05	0.71 \pm 0.25 P 0.05	0.38 \pm 0.166	0.16 \pm 0.136
		0.82 \pm 0.127 P 0.005	0.60 \pm 0.114 P 0.01	

TABLE II The influence of adrenaline (ADR), nicotinic acid (Nic ac) and Nic ac and ADR in combination on the FFA glucose and lactic acid content of the blood. Symbols as in table I

	ADR (I)	ADR + Nic ac (II)	Nic ac (III)	Mean difference in ADR effects with and without Nic. a I (II III)
FFA μ eq/l				
Basal values	686 \pm 73.6	638 \pm 106.3	583 \pm 62.8	
Mean change from basal values after \rightarrow 3 min		-226 \pm 68.1 P < 0.05	-258 \pm 38.3 P < 0.005	
+10	317 \pm 120.6	-278 \pm 98.9 P < 0.05	-334 \pm 41.5 P < 0.005	262 \pm 187.7
30	768 \pm 162.9 P < 0.01	-336 \pm 95.9 P < 0.05	396 \pm 47.9 P < 0.005	708 \pm 195.4 P < 0.05
45	661 \pm 187.5 P < 0.05	-360 \pm 97.6 P < 0.05	379 \pm 42.0 P < 0.001	642 \pm 183.8 P < 0.05
60	202 \pm 128.6	-434 \pm 111.8 P < 0.02	-367 \pm 45.1 P < 0.005	274 \pm 141.8
90	-192 \pm 93.6	-430 \pm 119.1 P < 0.05	-406 \pm 53.6 P < 0.005	-168 \pm 118.8
Glucose mg/100 ml				
Basal values	78 \pm 4.8	81 \pm 3.5	81 \pm 2.5	
Mean change from basal values after \rightarrow 3 min		8 \pm 6.0	3 \pm 1.3	
+10	17 \pm 2.7 P < 0.005	21 \pm 10.6 P = 0.05	5 \pm 1.5 P < 0.05	1 \pm 10.9
30	54 \pm 6.9 P < 0.005	50 \pm 7.5 P < 0.005	4 \pm 1.9	7 \pm 7.2
45	53 \pm 4.3 P < 0.001	57 \pm 12.9 P = 0.05	3 \pm 1.7	-1 \pm 9.6
60	46 \pm 2.9 P < 0.001	29 \pm 7.7 P < 0.05	0 \pm 2.8	17 \pm 8.3
90	26 \pm 4.5 P = 0.005	7 \pm 8.7	0 \pm 2.1	19 \pm 9.7
Lactic acid mg/100 ml				
Basal values	6.8 \pm 1.17	5.4 \pm 0.76	4.5 \pm 0.31	
Mean change from basal values after \rightarrow 3 min		0.2 \pm 0.65	0.3 \pm 0.48	
+10	1.7 \pm 0.51 P < 0.05	3.6 \pm 1.50	0.2 \pm 0.37	-1.7 \pm 1.63
30	8.0 \pm 1.04 P < 0.005	1.4 \pm 0.94 P = 0.05	0 \pm 0.49	2.2 \pm 0.99
45	5.5 \pm 0.58 P < 0.001	5.6 \pm 0.98 P = 0.005	0.9 \pm 0.61	0 \pm 0.85
60	2.5 \pm 0.53 P < 0.01	3.1 \pm 0.57 P = 0.01	1.2 \pm 0.41	-0.4 \pm 0.68
90	0.4 \pm 0.44	0.14 \pm 0.98	0.1 \pm 0.35	-0.4 \pm 0.73

Effect of nicotinic acid infusion Infusion of nic ac in a dose of 5 mg/min for 120 min provoked an initial flush which gradually disappeared. No definite change in the oxygen consumption was noted (Fig 1, Table I). The CO_2 elimination, on the other hand, began to increase about 30 min after commencement of the nic ac infusion. The respiratory quotient thereby increased from a mean basal value of 0.76 to a maximum of 0.82. The difference 0.06 ± 0.019 was significant ($P < 0.05$). The ventilation and CO_2 production increase ran parallel.

Nic ac reduced the plasma FFA concentration considerably, and after about 60 min this concentration reached a new level which lay approximately 350 $\mu\text{eq/l}$ below the initial value. The blood glucose concentration increased very slightly, while the blood lactate concentration showed no significant change (Fig 2, Table II). The pulse frequency increased initially by 10–15 beats/min but there was no alteration in blood pressure (Fig 2).

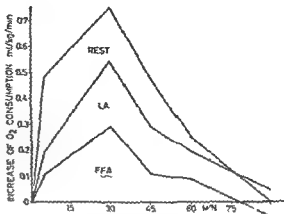
Infusion of nicotinic acid + adrenaline When the subject was given nic ac before and during the A infusion the adrenaline effect on the oxygen consumption was reduced (Fig 1, Table I), and this reduction was most pronounced at the end of the A infusion period, when in the subjects treated with A alone the plasma FFA concentration reached its maximum. In the nic ac treated subjects the elevating effect of A on FFA was completely inhibited (Fig 2, Table II).

The mean difference in A effect (0–45 min) on the O_2 consumption in tests on the same person without and with nic ac was 0.15 ± 0.038 ml/kg/min ($P < 0.02$, $n = 5$).

In Table I and II the sum of the separate effects of nic ac and A are also compared with the effect of these drugs in combination. A reduction of the O_2 consumption (0.15 ± 0.048 ml O_2 /kg min, $P < 0.05$) was shown when the summed mean values during the period 0–60 min were compared. On the other hand nic ac caused no reduction of the A induced increase in CO_2 production. The RQ were higher in the nic ac + A experiments than in the A experiments (mean values 0.94 and 0.87 after 10 min respectively). During A infusion, however, the RQ must be evaluated with some caution: part of the increased CO_2 production is probably due to the fact that CO_2 is driven out from the bicarbonate of the blood and thus owing to the increase in different acids (lactic acid and FFA) induced by the A infusion. This relationship has been studied in more detail by Lundholm and Svedmyr (1966a). In the nic ac – A experiments the plasma FFA increase was inhibited, however. It seems probably, therefore, that the RQ increase in these experiments as compared with the A experiments was a true one. In that case this should indicate an increase of the carbohydrate metabolism in the nic ac – A experiments. Some support for this assumption is also provided by the course of the blood glucose concentration, which decreased considerably more rapidly in the nic ac – A experiments after discontinuation of the A infusion than in the A experiments (Fig 2, Table II). The effect of A infusion on the blood lactate concentration was not influenced by the nic ac treatment.

The respiratory volume in the nic ac – A experiments showed approximately

Fig. 3 Tentative presentation of the relative importance of FFA and lactic acid (L.A.) metabolism for the calorigenic effect of A in man. FFA Part of the calorigenic effect of A which was blocked by nicotinic acid and attributed to increased FFA metabolism. L.A. Increase of oxygen consumption calculated from the relationship between the increase of lactic acid content in the blood and the stimulation of oxygen consumption as found by Svedmyr (1966 b). The hyperglycemic effect of A and its stimulation of the heart work may contribute to the Rest effect.



the same course as the CO_2 production, which substantiates previous findings (Lundholm and Svedmyr 1966 a) of a relationship between the respiration-stimulating effect of A and its influence on CO_2 production. The effects of the adrenaline on pulse and systolic blood pressure were not influenced by nic ac, but the reduction of the diastolic pressure was smaller after nic ac (Fig. 2).

Effect of L(+)-lactate on oxygen consumption and blood lactate concentration in man. Preliminary experiments indicated that on infusion of 2.3 mg L(+)-lactate/kg/min the blood lactate concentration increased to approximately the same degree as with adrenaline infusion. It is of interest that this lactate quantity agrees with the maximal lactic acid elimination from the forearm tissues which was induced by an adrenaline infusion in the same dose (Lundholm and Svedmyr 1966 b). On infusion of this quantity of L(+)-lactate the blood lactate concentration increased maximally by 9.4 mg% (8.0 after A) and the oxygen concentration by 10% (21% after A). These experiments are reported in more detail in another paper (Svedmyr 1966 c). When the increase in blood lactate concentration was correlated with the increase in oxygen consumption in these experiments the following regression equation was obtained $Y = 0.028X + 0.032$ Y = increase of O_2 consumption in ml/kg/min over the basal level and X = increase of blood lactate concentration (in mg% over the basal value). On calculating by means of this equation the increase in oxygen consumption that could be expected to be induced on the basis of increased blood lactate concentration on infusion of A, the curve in Fig. 3 was obtained. The area below this curve in the period 0–90 min after the start of the infusion was 35%, of that below the corresponding λ curve.

Discussion

FFA metabolism. After treatment with nic ac the calorigenic effect of A in man was reduced and at the same time its FFA mobilizing effect was selectively inhibited. The results agree with the findings of Havel *et al.* (1964) with regard to the calorigenic

effect of NA in man. In Fig. 3 the proportion of the calorogenic effect of A which was blocked by nic. ac. has been marked out by IIA. This proportion constitutes 29 % of the total effect in these tests. An important question is whether the blockade of the IIA metabolism had caused the relative inhibition of the calorogenic effect. This seems probable since nic. ac. did not inhibit any other effects of A, but there is no conclusive evidence. Had it been feasible by infusion of free fatty acids to raise the plasma level to the same degree as after the A infusion it might have been possible to study this question further, but free fatty acids are too toxic to be administered in this way (Oro and Wrethlid 1961), and if they had been given together with albumin the fluid volume infused would have been very large.

Assuming that there is a causative relationship between IIA mobilization and calorogenic effect, the stimulation of the oxidative processes might perhaps be interpreted as a result of increased access to substrate. Steinberg (1963) found in experiments *in vitro* that the oxidation of IIA increased with an increase of the IIA concentration in the medium. This was not accompanied by any increase in oxygen consumption, however, and this effect was therefore probably associated with reduced utilization of other substrates. It is well known that there is an inverse relationship between, e.g., the IIA and glucose utilization of cardiac muscle (Opie, Lyant and Ship 1961, Randle 1963). In my experiments, for example, nic. ac. reduced the plasma IIA concentration considerably under basal conditions without influencing the basal O_2 consumption. The R.Q. rose, however, indicating that the oxidation of carbohydrate and/or protein had increased (Challoner and Steinberg 1966). I have, however, recently found that an increased IIA concentration in the perfusate increased the O_2 consumption of isolated rat heart.

It has also been demonstrated that NA considerably increases the turnover rate of plasma IIA (Havel 1964, Steinberg *et al.* 1963). The increased efflux of IIA from plasma is also combined with an increase in the incorporation of IIA to glycerides. Adrenaline raises the blood glucose concentration, and it is well known that increase in this concentration increases the resynthesis of triacylglycerides (Steinberg 1963). NA should therefore stimulate an IIA cycle with an increase in both hydrolysis and re-synthesis of triacylglycerides. This cycle is energy-consuming and it has been suggested by Ball and Junjas (1961), Havel (1964) and Mavrou (1965) to be partly responsible for the calorogenic effect of CA. It has been reported that NA, in *in vivo* experiments, increases the triacylglyceride concentration in the liver, heart and muscle (Carlson, Liljedahl and Wærn 1965).

Further studies of this problem are, however, required in order to determine more quantitatively the relative importance of direct IIA oxidation and/or the resynthesis of IIA to triacylglycerides for the calorogenic effect of CA.

Lactate metabolism

An important question is whether a lactate infusion can completely emulate the effect exerted by A on the lactate metabolism. This question is closely connected with that of whether lactate production and lactate oxidation take place in different

tissues and how an increase in lactate metabolism induces stimulation of the oxygen consumption. According to the 'lactate cycle' theory of Cori (1931) A induces glycogenolysis in skeletal muscle after which the lactic acid produced is transported via the blood to the liver where it is to the greater part resynthesized to glycogen. The stimulation of oxygen consumption by the lactate would thus be due to energy losses on resynthesis of the lactate to glycogen. Adrenaline would thus stimulate both a 'lactate cycle' and a 'FFA cycle'. The doubling of the oxygen consumption of the liver and the increase of its uptake of lactate which has been demonstrated in man by Bearn, Billing and Sherlock (1951) during an A infusion could be explained, at least in part, by an increased lactate metabolism. NA also stimulated the oxygen consumption of the liver, — the effect was about one third as great as that of A — and therefore an increased FFA metabolism may have contributed to these effects.

If Cori's 'lactate cycle' theory is correct the effect of a lactate infusion should emulate fairly well that of A, on the lactate metabolism. More recent studies appear to indicate, however, that the lactate metabolism is more complicated than is outlined above.

In experiments on the rabbit Drury and Wick (1965) found that the major part (65 %) of infused C^{14} labelled L(+)-lactate is oxidized to CO_2 and that only a few per cent were found in the liver and muscle glycogen. In eviscerated animals the 'turnover rate' of radioactively labelled lactate was about 8 times slower than in animal with an intact liver. The animals were still able to oxidize lactate, however, though with greatly increased lactate concentrations in the blood. Even if the liver is the most important organ for lactate oxidation, it can also be metabolized to some extent by other organs such as heart, kidney and brain (Brin 1965, Levy 1965, Sacks 1965). It has not been shown, however, that lactate stimulates the oxygen consumption in these organs. However, in adipose tissue CA stimulates both the oxygen consumption, lactic acid production and FFA release, and lactate also stimulates the oxygen consumption of adipose tissue (Lynn McLeod and Brown 1960, Havel 1964). It is therefore possible that both lactate production and lactate oxidation may take place in combination with stimulation of the O_2 consumption in adipose tissue. If this is so then the lactate concentration in the tissues should not be completely reflected by the blood lactate concentration. Lactate diffuses relatively slowly and considerable concentration difference in lactate concentration between blood and tissues have been shown (Sacks 1937, Lundholm 1956). It is therefore possible that the lactate concentration in adipose and possibly other tissues was higher on infusion of A than of lactate. Such a possibility might have given rise to some undervaluation of the role of lactate in the calorigenic effect of adrenaline.

Schimassek (1965) has found that A and glucagon stimulated lactate uptake and O_2 consumption of the liver to a considerable extent. Laton and Park (1965) reported that phosphoenolpyruvate carboxylase had a rate limiting effect on the transformation of lactate to glucose by the rat liver. The activity of this enzyme was stimulated by glucagon, A and cyclic 3 — 5 AMP. It is therefore probable that A has a stimulating effect on the lactate uptake and resynthesis by the liver.

effect could partly explain the differences in the results of Cori (1931) and Drury and Wick (1965) with regard to lactate metabolism.

Other mechanisms As may be seen in Fig. 3 the total calorigenic effect of A was somewhat greater than the sum of the calorigenic effects that could be assumed to be due to stimulation of the FFA and lactate metabolism. It is possible that the role of the lactate metabolism was undervaluated and that the magnitude of this "residual effect" is not so great as is indicated in Fig. 3. It is probable, however, that other mechanisms than those discussed above may have been of contributory importance.

Adrenalin increased the heart frequency by about 10 beats/min (Fig. 2) and has also been found to increase the cardiac output by 50–100% (Goldenberg *et al.* 1948). The respiratory volume also increased. It is highly probable that these increased muscular effects may be associated with stimulation of the oxygen consumption, but with the data available at present it is difficult to exactly calculate their part in the calorigenic effect. The hyperglycemic effect of A is probably of some importance for its calorigenic action, too (Boothby and Sandiford 1923) and this will be evaluated in a further study. Adrenaline also increased the oxygen consumption of the forearm musculature in man (Lundholm and Svedmyr 1966b) and it has not as yet been established whether this effect was due to an increase in the lactate or FFA metabolism or induced by a more direct stimulation of the muscle metabolism.

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Electrophysiological and Micro-electrophoretic Studies on Sympathetic Preganglionic Neurons in the Spinal Cord

By

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Abstract

HONGO, T and R W RYALL *Electrophysiological and micro-electrophoretic studies on sympathetic preganglionic neurones in the spinal cord* Acta physiol scand 1966 68 96—104

Preganglionic sympathetic neurones in the thoracic cord of the spinal cat were located by recording antidromic extracellular potentials in response to stimulation of preganglionic fibres in the superior splanchnic nerve. Antidromic potentials were only recorded in the region of the intermediolateral cell column. Few units were firing spontaneously or could be orthodromically activated. The conduction velocities in the preganglionic axons of units from which recordings were obtained ranged from 1.5 to 9 m/sec. Local micro-electrophoretic applications of DL-homocysteic acid fired the cells but noradrenaline had no excitatory effect and did not inhibit spontaneous synaptic or antidromic firing or the firing to the excitant amino acid. Acetylcholine had no effect on the few cells tested.

Noradrenaline (NA) is a potent inhibitory substance when applied micro-electrophoretically to some interneurons in the lumbar region of the spinal cord of the cat (Engberg and Ryall 1965, 1966) and there was some correspondence between the distribution of sensitive interneurons and the relative densities of NA containing terminals in this region of the cord. This and other evidence led to the suggestion that NA is an inhibitory transmitter liberated from the terminals of descending fibres (Engberg and Ryall 1965, 1966). There is a high density of NA containing terminals in the intermediolateral column of the spinal cord (Dahlström and Luxe 1965) and the preganglionic sympathetic fibres are known to arise from cell bodies located in this region (see Mitchell 1953). It was therefore of interest to determine the sensitivity of the preganglionic sympathetic neurones to NA. When this investigation was carried out there was no published account of the electrophysiological properties of these neurones. However, Fernandez de Molina, Kuno and Perl (1965) have

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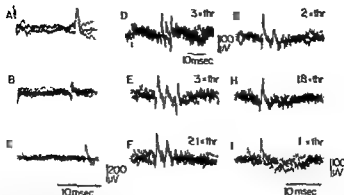


Fig. 1. Types of antidromic potentials recorded in the thoracic cord to antidromic stimulation of fibres in the superior splanchnic nerve. A: unitary negative potential. B: positive-negative potential, probably unitary. C: unitary negative-positive potential (stimulus straddling threshold). D–I: compound potentials produced from several units at different intensities of stimulation ranging from 1 to 3 times threshold for the first short latency potential (I). Note expanded sweep in E–I. In A–D the stimulus artifact occurs near the beginning of each sweep.

recently reported on the properties of neurones antidromically activated from the white ramus. Many of the present results with antidromic stimulation of the splanchnic nerve confirm their findings. Thus the present results will be presented in comparison with those of Fernandez de Molina *et al.* (1965), with special attention being paid to those observations which are complementary to those of the latter authors: a preliminary account has been published (Hongo and Ryall 1966).

Methods

Cats were operated upon under anaesthesia. The spinal cord was exposed from the upper border of the fifth thoracic segment down to the lower part of the ninth thoracic segment and the dorsal roots were cut ipsilaterally to the recording side. Recordings were usually made at the seventh thoracic segment and occasionally the central stump of the dorsal root of the same segment was used.

A neuromuscular blockade was maintained by i.v. injections of gallamine triethiodide. A pneumothorax was routinely employed. The majority of the experiments were carried out with 5-barrelled micro-electrodes which had overall tip diameters of 4–6 μ . The recording and electrophoretic techniques were the same as those used in a previous investigation (Engberg and Ryall 1966). However, some of the recordings were made with single-barrelled electrodes (tip diameter approximately 1 μ) filled with 3M sodium chloride. Square wave pulses of 0.3–0.5 msec duration were used for stimulation of the preganglionic fibres. Recordings of volleys in the peripheral stumps of the dorsal and ventral roots were usually made at the end of the experiment and the length of the splanchnic nerve was measured in order to determine the conduction velocities.

All extracellularly recorded potentials are shown with negativity upwards.

Results

Antidromically generated potentials

It was sometimes difficult to locate extracellular potentials which could be attributed to potentials generated in sympathetic neurones by antidromic volleys in the ipsilateral splanchnic nerve. This was probably due to the small size of the cells and their dendrites (Fernandez de Molina *et al.* 1965) and therefore of the poten-

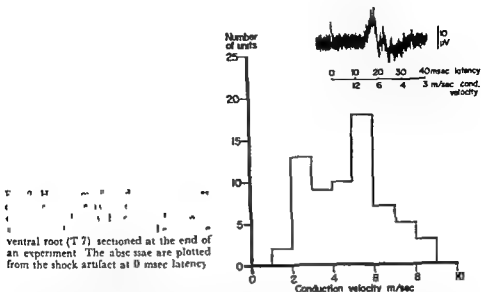
and to the variation in the size of the cell groups at different levels of the cord and in different cats.

Potentials were only considered to be antidromically generated if they satisfied the following criteria, 1) the latency from the stimulus artifact was invariable at a constant frequency of stimulation and was compatible with the speed of antidromic propagation of volleys in the preganglionic nerve, 2) units followed a reasonably high frequency of stimulation (at least 100/sec) compatible with unimpaired conduction of the antidromic volleys.

Focal potentials recorded from groups of cells were small and probably generated mainly by only a few cells. These potentials were always irregular in outline (Fig. 1 D) and were composed of several negative waves. These waves could be differentiated by adjusting the stimulus intensity, the units with the longest latency firing with the largest stimulus and *vice versa*. An example of such a focal potential is shown in Fig. 1 D—I. In I, the stimulus was just threshold for a unit responding with the shortest latency (17 msec) in the group. The first wave is probably due mainly to this unit since the size of the potential did not increase markedly when the stimulus intensity was increased. At 1.8 times threshold a second potential (latency 19.5 msec) became evident. This second potential increased stepwise with increase in stimulus intensity up to 2.1 times threshold for the first unit. At 2.0 times threshold a third small potential (latency 22 msec) may be seen, but this did not increase when the stimulus was increased and at 3.0 times threshold the third potential is partially masked by a fourth (latency 23.2 msec) which like the potential with the shortest latency appeared to be generated mainly by a single unit. Also of interest in these records is the small positivity preceding each negative potential but these were not always present.

The unitary spikes were often recorded in the absence of potentials from other cells (Fig. 1 A—C). They were usually predominantly negative and were far smaller (50–200 μ V, exceptionally 300 μ V) than those reported by Fernandez de Molina *et al.* (1965). The recording conditions were relatively stable; the same unitary spikes could be observed for more than 30 min. An initial positivity was frequently observed as seen in Fig. 1 B D—I which however appeared to have a gradual onset and was usually much smaller than the following negativity. Occasionally unitary spikes with a late positivity were also encountered (Fig. 1 C). The rise time and the duration of the unitary spikes as measured from the start of the negativity was 0.8 ± 0.2 (standard deviation, 61 determinations) msec and 2.3 ± 0.6 (58 determinations) msec respectively. The small late positivity (not always present) was too small in relation to the noise level to allow measurement.

Many other neurones were not antidromically activated from the ipsilateral splanchnic nerve but could be orthodromically activated by stimulation of the contralateral splanchnic nerve. The latencies of these orthodromic potentials were in the range 1.5–2.5 msec. The small late positivity (not always present) was too small in relation to the noise level to allow measurement.



and variable latencies were encountered occasionally. This was especially the case when the ipsilateral splanchnic nerve was stimulated in preparations in which the cord was not transected at both T₅ and T₉. These discharges of presumably synaptic origin probably represent an afferent inflow from lower segments of the cord, but the possibility of activation via axon collaterals of the preganglionic neurones cannot be excluded.

Latency of antidromic potentials

The antidromic axonal conduction velocities for sixty-seven units are plotted as a frequency histogram in Fig. 2. Also shown in Fig. 2 is a record of the small volley recorded in the peripheral stump of the ventral root when the splanchnic nerve was stimulated at the end of an experiment.

The range of velocities was from 1.5 to 9 m/sec (Fig. 2) which was similar to the distribution of conduction velocities of the antidromic volleys recorded in the ventral root (Fig. 2, inset record). The range is similar to that reported by Fernandez de Molina *et al.* (1965) who stimulated the white rami. Faster conducting volleys equivalent to the P1 subgroup (11–15 m/sec) as defined by these authors were not clearly found in the compound ventral root potentials in the present study.

Spontaneous firing and synaptic activation of preganglionic neurones

The majority of antidromically activated units did not fire spontaneously and could not be fired by stimulation of either the central end of the ipsilateral dorsal root or by stimulation of the contralateral splanchnic nerve with single shocks (contralateral dorsal roots intact). However, in contrast with the experiments of Fernandez de Molina *et al.* (1965) a few were found which could be fired synaptically or were spontaneously active with a low frequency of firing (less than 10/sec). Such spike discharges could be attributed to either spontaneously active preganglionic neurones or to units which were antidromically activated by the splanchnic nerve.

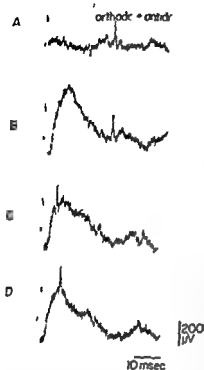


Fig 3 Extracellular spike potentials from a preganglionic neurone showing the collision between evoked orthodromic potentials and an incoming antidromic volley. In all records the splanchnic nerve was stimulated near the beginning of each sweep (indicated as artifact). The neurone fired antidromically at 26.5 msec latency (A, B) and was sometimes fired orthodromically by a dorsal root volley (C, D). In B, the dorsal root volley did not elicit a spike and the antidromic potential was seen with the same latency as in A. In C and D orthodromic potentials were generated and no antidromic potentials were observed. The axonal conduction velocity of this neurone was 3.1 m/sec.

ated unit by the similar sizes and shapes of the action potentials evoked spontaneously, orthodromically and antidromically, and, more conclusively, by the collision between the orthodromically and antidromically evoked spikes. This collision is illustrated in Fig. 3 for one neurone, which was activated with a variable latency by dorsal root stimulation. In records A–D the ipsilateral splanchnic nerve was stimulated antidromically near the beginning of each sweep. An antidromic potential with a latency of 26.5 msec (conduction velocity 3.1 m/sec) (A) was only observed when dorsal root stimulation failed to elicit a spike (B). Similarly, a collision between orthodromically propagating but spontaneously generated potentials and antidromic potentials was demonstrated on spontaneously firing units.

The location of the antidromic potentials

The records in Fig. 4 were obtained in one of two cats in which the area over which antidromic potentials could be recorded was relatively large compared with other experiments, although it was still small in absolute terms (approximately 300 μ in diameter). The area in which single unitary spikes were recorded was even smaller. However, antidromic potentials could sometimes be recorded over a distance of 200 μ . In every experiment the location of the electrode in the cord was determined by fixation and sectioning of the cord with the electrode tip *in situ*. Invariably, the antidromic potentials were only recorded from a small area in the intermediate-lateral region of the grey matter, as shown in Fig. 1. In this diagram the depth from the cord dorsum is only approximate since the degree of shrinkage which occurred when the cord was fixed and embedded was not known but was assumed

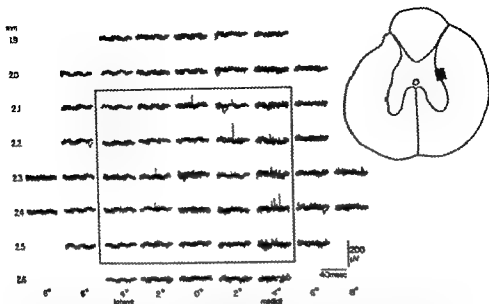


Fig. 4 Extracellular antidromic focal potentials recorded at different depths from the cord dorsum in tracks with the insertion spot kept constant but at different relative angles. The antidromic potentials were only observed within the area indicated by the square. The area of this square is indicated by the shaded area in the diagram to the right of the figure. The depth of the micro-electrode tip from the cord dorsum is indicated in mm and the relative tracking angles of the micro-electrode are shown below the figure.

to be about fifteen per cent. Inspection of stained histological sections showed that the size of the intermedio-lateral horn varied markedly at different levels along the axis of the cord and even at the same level on opposite sides of the spinal cord. This variation probably explains the difficulty in locating the preganglionic neurones, and the low cell density may to some extent explain the small focal potentials. Furthermore most of the cells in the intermedio-lateral column were very small (less than 20μ in diameter).

The action of noradrenaline on sympathetic preganglionic neurones

NA was administered micro-electrophoretically with currents up to $80-100 \mu A$ through a $1 M$ solution of the bitartrate salt while recording antidromic unitary potentials from twelve neurones. In no instance was there any effect on the antidromic firing. This shows that NA is not a particularly potent inhibitory substance on these cells. Furthermore excitation was never observed. Antidromic firing may not be a very sensitive test for a slight inhibitory action because of a high safety factor for antidromic invasion. Spontaneous firing should be a more sensitive index but was rarely observed. However NA was tested on three occasions on cells which were firing spontaneously and had spikes sufficiently large ($100-300 \mu V$) to allow discrimination from the background noise. Again no effect was observed with noradrenaline. On two cells the activation of the preganglionic

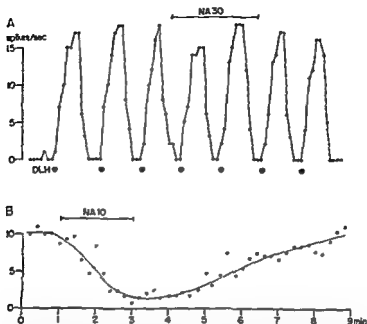


Fig. 5. A: effect of NA on the firing to DLH of a sympathetic preganglionic neurone. DLH was ejected from the micro-electrode for 30 sec at each dot. NA (30×10^{-6} A) was applied during the period indicated by the horizontal bar. B: effect of NA on the synaptic firing of another unit (no stimulation of contralateral splanchnic nerve (contralateral dorsal root intact). This unit was not a preganglionic neurone. The contralateral nerve was stimulated at a frequency of 4 sec and each stimulus produced 2 or 3 spikes. The numbers of spikes in each second (averaged over 12 sec) are plotted as ordinates in the figure.

neurone from the dorsal root was also unaffected. Quiescent preganglionic neurones could be fired by the micro-electrophoretic application of an excitant amino acid, DL-homocysteic acid. Fig. 5A shows the effect produced by repeated brief ejections of this substance on the firing of a neurone which was invaded antidromically from the splanchnic nerve. During the period indicated by the horizontal bar, NA was also ejected with a current of 30×10^{-6} A but it neither excited the cell nor did it reduce the firing to the amino acid. However, it was only possible to carry out such an examination on three cells since it was usually difficult to maintain the size of the extracellular potentials constant for a sufficiently long period. This inability to maintain the spike size constant was partly due to the fact that small movements of the spinal cord in relation to the micro-electrode had a marked effect on the spike amplitude: such movements were often troublesome, despite the routine use of a pneumothorax, particularly towards the end of a long experiment when pulsating movements of the cord became more prominent. Another difficulty was that the excitant amino acid often caused a rapid decrease in spike size in the concentrations required to fire the cells.

In contrast, many other types of neurone may be inhibited by NA applied micro-electrophoretically (Engberg and Ryall, 1965, 1966). The neurone in Fig. 5B was activated by stimulation of the contralateral splanchnic nerve at a frequency

of 4/sec, but was not invaded antidromically from the ipsilateral nerve and lay dorsal to the intermedio lateral region. Each stimulus produced a discharge of one to three spikes and the total number of spikes in one second (averaged over 12 sec) are plotted as ordinates in the figure. During the application of NA with a current of only 10×10^{-8} A the synaptic discharge was virtually abolished, and when the application ceased there was a slow recovery.

Acetylcholine was tested by micro-electrophoretic administration on four preganglionic neurones, but was inactive.

Discussion

The electrophysiological studies reported in this paper confirm and extend those of Fernandez de Molina *et al* (1965). In the present investigation the extracellularly recorded potentials were usually small and predominantly negative. They were usually stable enough to allow observations for a considerable time. In contrast, Fernandez de Molina *et al* (1965) obtained large (up to 15 mV) and usually positive extracellular potentials, or »giant extracellular potentials» (Granit and Phillips 1956, Freygang 1958, Freygang and Frank 1959). The duration of the unitary spikes obtained in the present study (2.3 ± 0.6 msec) was considerably shorter than that reported by these authors. This is not unexpected since the recording conditions are clearly different: it is very likely that the tip of the micro-electrode was positioned in close proximity to and probably invaginating the cell membrane in their study. However, it should be noted that the duration of the extracellular negative potential of the sympathetic preganglionic neurones is still long. The duration of the equivalent extracellular potential in other neurones is about 1 msec in spinal motoneurones (Fatt 1957, Nelson and Frank 1964) and 0.48 ± 0.16 (standard deviation, 18 determinations) msec in neurones of the spinocervical tract (Hongo and Lundberg unpublished).

Since antidromic potentials were only recorded from the intermedio-lateral region of the grey matter there was no evidence to support the suggestion that there may be another group of preganglionic neurones located more medially (see Mitchell 1953). There was a variation in the size of the area from which antidromic potentials were recorded along the axis of the cord but no attempt was made to correlate this observation with variations in the size of the intermedio-lateral cell column, which were seen histologically.

Fernandez de Molina *et al* (1965) found no unit which could be orthodromically activated and in the present investigation only a few preganglionic neurones could be activated orthodromically by stimulation of dorsal root fibres. These extracellularly recorded orthodromic potentials were positively identified as belonging to the preganglionic neurones by colliding orthodromic and antidromic volleys. The conduction velocity of axons in these units did not belong to the P₁ subgroup (11–15 m/sec) as was suggested by Fernandez de Molina *et al* (1965). Since so few units were orthodromically activated it seems likely that only a small proportion of the preganglionic neurones can participate in segmental autonomic

reflexes in this preparation. It is possible that in the spinal preparation the segmental reflex pathways to the preganglionic neurones are partially inhibited.

The failure of NA to affect antidromic firing of the preganglionic neurones is evidence that it was not a powerful inhibitory substance. There was, in addition, no evidence for an excitatory action. Unfortunately, spontaneous or synaptic firing, which should be more sensitive tests for inhibition, was rarely observed in these preparations, but was unaffected by NA on the few occasions when tests were possible. Similarly, firing by an excitant amino acid was also unaffected by NA. With the techniques used in this investigation it was possible to show that NA has marked inhibitory actions on many lumbar interneurons (Ryall 1965, Engberg and Ryall 1965, 1966) and on interneurons in the thoracic cord which were fired by afferents in the splanchnic nerves. Thus the failure to affect the firing of the preganglionic neurones with NA is unlikely to be due to technical difficulties in administration.

These studies have shown that NA has no action on at least some of the cells giving rise to preganglionic fibres in the splanchnic nerve. Thus the results suggest that the NA terminals in the intermediate lateral column of the thoracic cord (Dahlstrom and Fuxe 1965) are ending on neurones which are not those giving rise to the preganglionic fibres in the splanchnic nerve. The possibility still remains that the noradrenergic innervation occurs on preganglionic neurones synapsing in the sympathetic trunk ganglia.

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Glucose Uptake in Potassium-Depolarized Mammalian Muscle

By

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Abstract

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Experiments were performed on perfused skeletal muscle from the rat. The glucose uptake was measured by the method of Crone (1965). The effect of potassium concentration on the glucose uptake was studied. The results are summarized in the following table.

The striated muscle cell displays a clear selectivity to the entry of non-electrolytes. Some molecules enter the cell interior faster than others of comparable size and physical properties — probably due to interaction with components in the cell membrane. Glucose is an example of a hydrophilic substance which enters faster than do other hexoses. The nature of the interaction between cell membrane and permeant molecule is completely unknown and therefore, it has so far been almost impossible to elucidate the mechanism by which insulin enhances the rate of passage of glucose into striated muscle.

In order to throw some light on the nature of the facilitated passage of glucose experiments were performed in which the effect on the muscular glucose uptake of increasing the extracellular potassium concentration was studied. The idea behind the work was originally that the depolarization as such might increase the permeability of the membrane to glucose, but the work later focussed on the possible connection between the glucose uptake and the processes in the cell membrane responsible for sodium extrusion and potassium uptake.

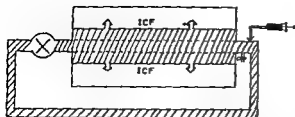


Fig 1 Diagram of experimental set up. Blood diluted with cat Ringer is circulated by a pump. Material passes from the extracellular space into the intracellular space (ICF). Glucose is continuously added by means of a motor-driven syringe.

The general plan for the experiments was to determine both the uptake of glucose and the oxygen consumption in an isolated perfused cat hind limb before and after addition of potassium and in other experiments to see whether interference with ion transport by addition of strophanthin affected the glucose uptake.

Methods

Experimental technique and calculations 61 expts. were performed on cats starved for 18 hrs. Usually

a bubble oxygenator with a gas mixture (saturated with water vapour) containing 5.6% CO_2 and oxygen. The blood was circulated with a Sigma motor pump. The composition of the Ringer solution added to the blood in the control period was (meq/l): Na 150, K 4.1, Mg 2.6, Ca 1.8, Cl 130, HCO_3^- 24, H_2PO_4^- 1.5, SO_4^{2-} 2.6. When potassium rich solution was used sodium chloride was substituted with potassium chloride.

In the earlier experiments Rheomacrodex[®] was added to provide the necessary colloid osmotic pressure but as it was found that this substance greatly increased the inulin blank value it was given up and the potassium rich perfusate did not thereafter contain high molecular material. Generally one third of the original perfusate was substituted with potassium rich perfusate so that the protein concentration in the mixed blood only fell by 30%. The initial potassium concentration after the substitution was about 3.5–4.5 mM.

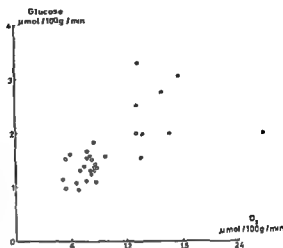
Fig 1 shows schematically the experimental set up. Glucose was infused continually in amounts corresponding to the uptake so that the concentration in the extracellular fluid remained almost constant during the control period. The uptake was calculated from the continually infused amount of glucose. When the concentration fell or rose the product of the difference in glucose concentration times the extracellular space was added to or subtracted from this value.

The oxygen consumption was calculated as the arterio-venous difference times the blood flow. It was determined once in the control period and twice or three times in the potassium period. The RQ was determined as the ratio between the CO_2 content and O_2 content in the blood sample drawn in the control period. The extracellular space was determined with inulin after 1 1/2 hrs equilibration.

1400 ml. The strophanthin doses were between 0.1–0.3 mg except in a few experiments in which either doses of 2.0 mg or of 3 mg were tried with no obvious difference in effect.

The membrane potential of single muscle fibres was measured in some of the experiments by means of a micropipette filled with 3 M KCl. Pipettes with a tip potential above 10 mV were not used. The pipettes were filled according to the desaturation procedure described by Caldwell and the signal from the pipettes was recorded on a oscilloscope and the depth of the signal was inverted.

Fig 2 Glucose uptake and oxygen consumption. Open circles: Control periods. Filled circles: Experiments with increased plasma potassium concentration. Every point represents average values from one experiment.



muscle (Nadel & J) Insulin was determined by means of Bojesen's colorimetric procedure (1954). Lactic acid was analyzed enzymatically as described by Lundholm, Mohr-Lundholm and Vamos (1963).

Results

1 Experiments with potassium depolarization

a *Control period* The hind limb was perfused for 1–1.2 hrs before potassium-rich blood was substituted. The results from 24 expts. in which both the glucose uptake and the oxygen consumption was determined are shown in Fig. 2. The glucose uptake is plotted as a function of the oxygen consumption.

The average glucose uptake was $1.4 \mu\text{mole}/100 \text{ g}/\text{min}$ (S.D. 0.25, $n = 24$). The oxygen consumption was $8.1 \mu\text{moles}/100 \text{ g}/\text{min}$ (S.D. 1.6, $n = 24$). The R.Q. was 0.75 (S.D. 0.15, $n = 24$). The low R.Q. shows convincingly that substrates other than glucose were used.

The glucose uptake in muscle depends among other factors on the plasma concentration of glucose as shown by Lundsgaard, Nielsen and Orskov (1939). This point was reinvestigated and it was found that when the glucose concentration went up from about 100 mg/100 ml to 300 mg/100 ml the uptake rose from 1.4 to 1.7 $\mu\text{moles}/100 \text{ g}/\text{min}$. As the glucose concentration often rose immediately after the substitution with potassium it is of importance to know that a rise in concentration of about 20–30 mg/100 ml has only a very limited effect on the uptake.

The plasma concentration of potassium stayed almost constant in the control period, a small loss of potassium led to an increase of about 1 mM during one hour's perfusion. The lactic acid concentration was also constant during this period, being

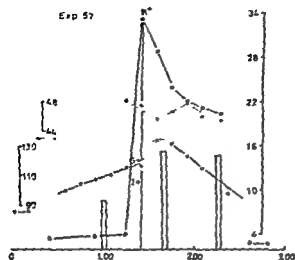


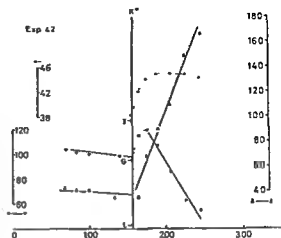
Fig 3 Variations in plasma concentration of glucose (●—●) potassium (○—○) and inulin (+—+) during a continuous infusion of 4 mg glucose per minute before and after potassium substitution. The vertical line labelled K shows time of substitution with potassium rich blood. I and G inulin and glucose concentration in potassium rich blood used for substitution. Glucose mg/100 ml Inulin mg/100 ml Potassium mEq/l. The vertical bars show the oxygen consumption. The oxygen consumption in the control period was 6.6 μmol/100 g/min. Abscissa: hours.

between 20 and 30 mg/100 ml. If anything there was a slight tendency to a fall. Fig 3 shows the concentration of glucose and potassium in an experiment which is representative.

b. Potassium depolarization. When part of the perfusion fluid was substituted with potassium-rich blood the potassium concentration rose within 2–3 min to an initial level of 35–45 mEq/l. During the subsequent 1–2 hrs the potassium concentration fell and levelled at 20–25 mEq/l. Fig 3 shows the time course of the potassium concentration after substitution.

The substitution with potassium-rich blood was accompanied by an increase in glucose uptake. The uptake rose from an average of 1.5 μmoles/100 g/min to 3.0 (S.D. 1.00, $n = 20$, an average increase of 100%). The steady fall in glucose concentration in the potassium-period compared with the slightly rising concentration in the control period is clearly seen on Fig 3 which also shows an initial sharp rise in glucose concentration which often followed substitution with potassium-rich blood. This early period lasted for 10–15 min and was succeeded by a period of 1–1.2 hrs with increased glucose uptake. There was no apparent connection between the increased glucose uptake and the potassium concentration at the rather high potassium concentrations which were established in these experiments. The early rise in glucose concentration was undoubtedly due to a rapid shrinkage of the extracellular space as revealed by a simultaneous rise in inulin concentration. Inulin was added to the substituting potassium-rich blood so that the concentration was close to that at the end of the control period. A rise in inulin concentration after the substitution, therefore, signified shrinkage of the extracellular space. The shrinkage was more or less complete after 20–30 min. During this period the muscle was gaining KCl until the internal [K]⁺[Cl]⁻ product equals the external value. The typical variation in inulin concentration is shown on Fig 4. The average rise in inulin concentration after the substitution was 18.3% in 10 expts in which there was a good correspond-

Fig 4 Variations in plasma concentration of glucose (●—●) inulin (+—+) and lactic acid (▲—▲) before and after potassium substitution. The vertical line labelled K shows time of potassium substitution. I G and L Inulin, glucose and lactic acid concentration in potassium rich blood used for substitution. Inulin mg/100 ml Glucose mg/100 ml Lactic acid mg/100 ml Glucose was continually infused (4.4 mg/min). Abscissa: hours



ence between the inulin concentration in the two portions of blood which were mixed. As the relative rise in glucose concentration in the early period was of the same magnitude as the rise in inulin concentration it is most probable that there was no reduction in glucose uptake in the very early phase. In many experiments the glucose concentration already began to fall during the early period of shrinkage of the extracellular space.

The increased glucose uptake occurred together with an increased oxygen consumption which was $16.5 \mu\text{moles}/100 \text{ g}/\text{min}$ (S.D. 4.4, $n = 8$). The results are shown in Fig. 2. The increase was thus of the same order (about 100%) as the increase in glucose uptake although the values scatter much more. In one experiment (which is not included in the calculation of the average) the oxygen consumption rose to $39.8 \mu\text{moles}/100 \text{ g}/\text{min}$ — more than 200% of the resting consumption. It was regularly observed that the oxygen consumption fell in the first 10 minutes after elevation of the potassium concentration. The reduction was about 30%.

The increased oxygen consumption lasted for the whole period of observation (1–2 hrs) which makes it unlikely that the rise was caused by contracture due to the potassium-depolarization. The muscle tension was not measured so that it is not possible to decide definitely whether there was actually a slight contracture. It is however, improbable that the increased oxygen consumption was due to contracture. Pillat, Kraupp, Giebisch and Stormann (1958) who perfused isolated cat hind limbs did not find any changes in the mechanical tension of the gastrocnemius muscle when the potassium concentration was increased to 20 meq/l. A reason for a lack of contracture in potassium depolarization under these circumstances was possibly that the potassium concentration outside the fibres only rose slowly, as reflected in the time-course of the depolarization. Hill and Howarth (1957) observed twitching in the frog sartorius when the potassium concentration was suddenly increased to 18 meq/l. The twitching came to an end within 2 min. When the potassium concentration was altered more gradually, no detectable twitching occurred. The rat



Fig. 5 Time course of change of membrane potential in a muscle cell when part of the perfusate was substituted with potassium rich blood at 0-time. The microelectrode was kept in position during the whole period of measurement.

of depolarization was measured in several of the present experiments and it took about 2 minutes to build up a new potassium concentration outside the cells. Fig. 5 shows the temporal course of the rate of change of membrane potential in one experiment. The fact that the intracellular micropipette remained in place during the period of potassium substitution speaks also against development of contracture in the muscle fibres.

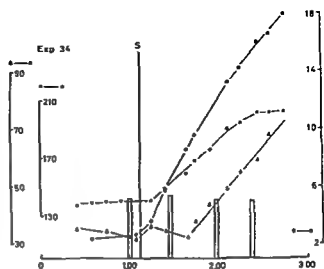
Fluid shifts accompanying potassium substitution. As already mentioned shrinkage of the extracellular space took place immediately after potassium substitution. As judged from the rise in inulin concentration (Fig. 4) the extracellular space was reduced by 20%. The extracellular space averaged 300–350 ml so that a volume of 60–70 ml moved into the cells. The intracellular space was not measured, but Huycke and Kruhnoff (1955) who used a similar preparation found a total water space of 900–1400, the intracellular volume would thus be of the order of 600–1000 ml. The degree of cellular swelling could therefore not be above 10%. In order to see whether this swelling was responsible for the increased uptake of glucose, experiments were performed in which SO_4^{2-} was the dominant anion, but the effect on glucose uptake and on oxygen consumption was unchanged.

2 The effect of strophanthin poisoning

The experiments reported so far only prove that potassium depolarization in mammalian muscle leads to an increase in glucose uptake which runs more or less in parallel with an increase in oxygen consumption. The rise in glucose uptake could be secondary to the increase in oxidative metabolism. Another possibility is that the transfer of glucose to the intracellular phase could somehow be coupled to the sodium extrusion mechanism; it is known that potassium depolarization leads to an increased efflux of sodium ions (Horowitz and Gerber 1965 a and b). A third possibility is that the extra influx of glucose could be a consequence of an increased membrane permeability in the depolarized state.

To differentiate between these possibilities experiments were carried out in which

Fig 6 Experiment with strophanthine poisoning 8 mg strophanthine was added ■ S
 ●—● plasma glucose concentration (mg/100 ml)
 ○—○ plasma potassium concentration (mEq/l)
 △—△ concentration of lactic acid (mg/100 ml) The vertical bars show the oxygen consumption The oxygen consumption in the control period was $7.8 \mu\text{mol}/100 \text{ g/min}$ Absc 333 hours



the sodium extrusion mechanism was inhibited with strophanthine. As usual the perfusion started with a control period lasting 1—1 1/2 hrs. Then strophanthine was added to give a final concentration of 10^{-5} M in the total water phase. After a time lag of 10—20 min a pronounced inhibition of the glucose uptake took place as reflected in a sharp rise in the extracellular concentration of glucose. Fig 6 shows the results from an experiment of this type.

The average uptake of glucose after strophanthine fell to $0.3 \mu\text{mole}/100 \text{ g/min}$ (S.D. 0.20, $n = 13$). The oxygen consumption was slightly depressed, being $6.9 \mu\text{moles}/100 \text{ g/min}$ (S.D. 2.65, $n = 12$) compared with an average uptake in the control period of $7.3 \mu\text{moles}/100 \text{ g/min}$. Fig 7 (open circles) shows the relation between glucose uptake and oxygen consumption in the strophanthine experiments. It is seen that the glucose uptake was inhibited to a much larger extent than the oxygen consumption.

The muscles began to lose potassium within 10 min after the addition of strophanthine (Fig 6). The average loss of potassium from the cells in 13 strophanthine experiments was $4.2 \mu\text{eq}/100 \text{ g/min}$ (S.D. 0.80, $n = 13$) as calculated from the rise in potassium concentration and the known value of the extracellular space. The potassium loss occurred together with a gain of sodium of the same magnitude indicating that the oxygen uptake fell by $0.4 \mu\text{mole}$ under circumstances in which the sodium pump was blocked to the extent that about $4.2 \mu\text{eq Na}$ were gained per 100 g per min. Thus in mammalian muscle 5 Na ions are pumped for each oxygen equivalent and only a small fraction of the oxygen uptake in mammalian muscle seems to be needed for processes concerned with sodium pumping.

3 Potassium depolarization after strophanthine

When potassium depolarization was induced in the usual way after strophanthine administration the strong inhibition of the glucose uptake was eliminated. The

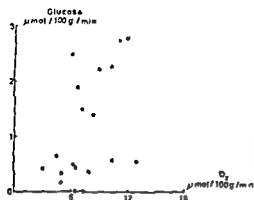


Fig 7 Glucose uptake and oxygen consumption. Open circles after addition of strophanthine. Filled circles experiments with potassium depolarization after a period with strophanthine.

TABLE I Glucose uptake and oxygen consumption in perfused cat muscles under varying circumstances

	Glucose uptake $\mu\text{moles}/100\text{ g}/\text{min}$	O_2 -consumption $\mu\text{moles}/100\text{ g}/\text{min}$
Control	1.4 (S.D. 0.25, n = 24)	8.1 (S.D. 1.60, n = 24)
K-depolarization	3.0 (S.D. 1.00, n = 20)	16.5 (S.D. 4.40, n = 8)
Strophanthine 10^{-6} M	0.3 (S.D. 0.20, n = 13)	6.9 (S.D. 2.6, n = 12)
K-depolarization after Strophanthine	2.1 (S.D. 0.50, n = 7)	8.5 (S.D. 2.06, n = 7)

glucose uptake went up to an average value of $2.1\text{ }\mu\text{moles}/100\text{ g}/\text{min}$ (S.D. 0.50, $n = 7$). This is somewhat higher than the value in the control series ($1.4\text{ }\mu\text{moles}/100\text{ g}/\text{min}$). The oxygen uptake under these circumstances was only moderately increased (Fig. 7), the average being $8.5\text{ }\mu\text{moles}/100\text{ g}/\text{min}$ (S.D. 2.06, $n = 7$). Generally, potassium acts as if it overcomes the effect of strophanthine, but the usual high glucose uptake and oxygen consumption found after potassium depolarization in preparations which had not received strophanthine were not seen.

Table I summarizes the results of the various types of experiments described in this and previous sections.

Increased vascular resistance. Hind limb perfusions can under normal circumstances continue for 4–5 hrs without any change in perfusion resistance. When the potassium concentration was elevated the perfusion resistance always went up, as an immediate response. After a few minutes the resistance began to fall again reaching the control level within $1/2$ –1 hr. The perfusion pressure rose from the normal level of 80–100 mm Hg to about 200–250 mm Hg. Addition of strophanthine also had this effect on the vascular resistance though the rise tended to be smaller. Again the resistance

fell slowly, but in contrast to the potassium experiments it never returned to normal values. When potassium depolarization was induced in strophanthin poisoned muscles a secondary rise in perfusion pressure took place. The fall was now very slow and the vascular resistance always ended at a much higher level than in simple potassium or strophanthin experiments.

The potassium dependent rise in perfusion pressure was preceded by a very small and rapid fall in pressure immediately after the addition of potassium (lasting for 1/2 min). This 'relaxation' was never seen preceding the 'strophanthin' rise.

Lactic acid. Both potassium depolarization and strophanthin administration led to an increase in lactic acid production. Lactic acid production was a constant feature in the strophanthin experiments where the rate of lactic acid production (as judged from the accumulation in the extracellular fluid) was $1.89 \mu\text{moles}/100 \text{ g/min}$ (average of 10 expts). The lactic acid production started with a time lag of 20–30 min (Fig. 6) which would imply that the lactic acid production was not a result of the arteriolar concentration (which was most pronounced immediately after the addition of strophanthin). The same argument applies to the lactic acid production observed during potassium-depolarization where the lactic acid production went on more or less unaffected by the fall in perfusion pressure which occurred after the initial rise. In some of the experiments the arteriolar contraction only lasted for 10–15 min and yet the lactic acid production seemed to continue. The lactic acid production in 9 potassium expts. averaged $2.2 \mu\text{moles}/100 \text{ g/min}$. The production of lactic acid was not seen in all experiments and it never became possible completely to control this parameter.

Discussion

The results reported in the previous section showed that it was possible to alter the rate with which glucose passes into muscle cells in various ways. The main observations were that the glucose uptake rose parallel with a rise in oxygen consumption when the external potassium concentration was increased, and that strophanthin reduced the glucose uptake considerably without leading to a corresponding fall in oxygen consumption.

Any interpretation of these results may place the cause either in the cell interior or in the cell membrane. If the increased cellular metabolism was responsible for the observed variations in the glucose flux then the mechanism might be that increased glucose metabolism in the potassium experiments led to a fall in the concentration of glucose-6-phosphate. This substance inhibits hexokinase and therefore phosphorylation would increase. An increased intracellular phosphorylation could diminish the concentration of free glucose inside the cell and thus increase the transfer rate.

The strophanthin experiments suggest another interpretation of the potassium effect on glucose uptake. Strophanthin inhibits the active sodium transport as reflected in the steady loss of potassium from and increased net uptake of sodium in the cells. The marked difference in the strophanthin effect on glucose uptake and oxidative metabolism makes it clear that glucose uptake is not necessarily a simple function

of the cellular metabolism and it is tempting to look at the inhibition of glucose uptake as being fundamentally related to the inhibition of the active transport of sodium (or active uptake of potassium). The rate of accumulation of potassium in the extracellular fluid averaged $4.2 \mu\text{eq}/100 \text{ g/min}$. As the volume of fluid outside the cells did not change the potassium loss corresponds to the net gain of sodium. If strophanthidin leads to no change in the passive fluxes of sodium or potassium (and this is probable as the membrane potential does not change under the influence of strophanthidin) the rate of sodium accumulation represents the inhibited part of the pumped sodium efflux. In the same period the glucose uptake fell by $1.2 \mu\text{moles}/100 \text{ g/min}$ so if there is a stoichiometric relationship between ion transport and glucose uptake the results may suggest that 1 molecule of glucose is taken up whilst 3–4 Na ions are pumped out (or alternatively, 3–4 potassium ions are pumped inwards).

The possibility that the influx of a metabolically important non electrolyte is coupled to the ionic transport in the membrane is interesting because in this way the influx of glucose could automatically be geared to the needs of the cell. Namely when muscle is activated the pumped sodium efflux increases in the recovery phase following an action potential (when the extra amount of sodium gained during the spike is pumped out). If the proposed mechanism is correct glucose influx would thus be coupled to the action potential which in itself is responsible for reactivation of the contractile energy requiring system in the cell interior. This mechanism would adjust glucose influx to the cellular energy requirements under circumstances where the need for glucose suddenly rises considerably. In this connection it is relevant to mention that Holloszy and Narahara (1965) found an immediate rise in the uptake of a carrier transported non metabolizable sugar 3 methylglucose in electrically stimulated frog sartorius.

The idea that there is a connection between ion transport and glucose transport has received considerable attention since the report of Riklis and Quastel (1958) in which it was shown that the presence of Na ions is necessary for the intestinal absorption of glucose. Crane (1962) proposed that glucose penetrates into the intestinal epithelial cell as a Na glucose complex. Na is then pumped out in the lumen again and thus the sodium pump drives the glucose transport. Schultz and Zalusky (1961) advanced a similar hypothesis. Czaky (1963) showed that strophanthidin inhibited glucose absorption in intestinal epithelium but he explains the Na sugar interaction in a different way regarding Na as being essential for that part of the transport system which converts chemical energy into pump activity. Because it is necessary for the possible ATPase system sodium could be involved in uphill transport of glucose. Sodium transport may be necessary for the transport of other substances than glucose thus Brndt and Beechwood (1965) found that the renal absorption of urate was strophanthidin sensitive and Vogel, Lauterbach and Kröger (1965) demonstrated the importance of sodium for the renal transport of glucose and of para-amino-hippuric acid. In one respect the situation in muscle is different from the above mentioned tissues as the glucose transport in this tissue is of an equilibrating type whilst in intestine and kidney it is of an uphill type.

Clausen (1965) Sotringen *et al.* Clausen (1965) did not find any inhibition effect any inhibitory effect on isolated rat diaphragms with strophanthin in concentrations between 10^{-2} — 10^{-6} M. He found on the other hand a correlation between sodium concentration in the incubating medium and glucose uptake in isolated rat diaphragms. His conclusion was, therefore, that glucose entry may be a function of the passive influx of sodium. This mechanism, however, cannot explain the increased glucose uptake in potassium depolarized muscle as the Na^+ influx is not altered under these circumstances (Horowitz and Gerber 1963a).

The interpretation given above is only one of several possible. Another is that the membrane potential as such controls the glucose uptake as it does with sodium efflux (Horowitz and Gerber 1965 a and b). The results of the strophanthin experiments do not easily fit such a concept because the membrane potential does not change immediately as a result of the strophanthin administration. Indirectly, however, the membrane potential may — by its control of sodium efflux — affect the glucose uptake.

In the third type of experiments described in the previous section potassium administration after strophanthin poisoning led to a significant increase in glucose uptake as if the strophanthin effect had thus been eliminated. The glucose uptake increased to levels somewhat higher than the resting uptake with only a small increase in oxygen consumption (another situation in which these two parameters do not follow one another). Glynn (1957) found that in red cells potassium could overcome the strophanthin effect. Page, Goerke and Storm (1964) demonstrated competition between strophanthin and potassium in cat heart muscle and the same appears to take place in muscle cells leading to the restoration of the ability to transport glucose. During the strophanthin period the muscle cells gain sodium so that the activity of the sodium pumping system may be greater once the strophanthin effect is overcome by potassium — this may explain that the glucose uptake was higher than in the control periods. A more detailed analysis of this type of experiment is however not possible as long as the ion fluxes are unknown.

Two other observations made during the experiments require comments.

1) Strophanthin always caused arteriolar constriction soon after administration (despite the fact that the metabolic effect of strophanthin always required some time to develop). Mason (1963) found that strophanthin reduced forearm blood flow due to an increased vascular resistance and Waldhausen and Herendeen (1963) showed that the renal blood flow reduction in *in vivo* experiments was due to strophanthin because of an increased vascular resistance. It has thus been proved in several cases that strophanthin can lead to contraction of smooth muscle presumably due to an increased spike activity (Casteels 1963).

2) The observation that potassium depolarization enhances the production of lactic acid was originally made by Hegrauer, Lenn and Clabb (1934) in isolated frog muscle and later confirmed by Hill and Howarth (1955) and by Muller and Simon (1960) who also used *in vitro* system. An increased production of lactic acid was found in the majority of the experiments reported in this article. It cannot be stated with

certainly whether this lactic acid production was due to the vasoconstriction which followed the potassium depolarization or whether it was due to other causes. Among these a reduction of intracellular ATP concentration could be the essential mechanism (due to uncoupling of oxidative phosphorylation). Briner, Simon, Frater and Tasker (1959) found a reduced ATP content in muscle soaked in Ringer solutions with 20 mM potassium. A lowered ATP concentration can lead to increased glycolysis because it is itself an inhibitor of phospho fructo-kinase.

If the theory of a coupling between glucose transport and ion transport is correct then the old theory of phosphorylation as an intermediary step in glucose transport gains a new significance. It has been argued that phosphorylation-dephosphorylation cannot be a necessary step in the transport because the hydroxyl-group specificity in active sugar transport is incompatible with the phosphorylation capacity of known kinases. If, however, it is the sodium/potassium transport system which requires phosphorylation-dephosphorylation (Skou 1964) then glucose transport is connected with phosphorylation in a way which does not imply phosphorylation of the sugar molecule.

It is generally accepted that there are three known causes of increased glucose uptake in muscle cells: insulin, muscular work and hypoxia. To this list high external potassium concentration must now be added. Future work will show whether the relation between ion transport and glucose transport will lead to a deeper understanding of the way in which glucose enters the muscle cell. Thus, the effect of insulin on glucose uptake might be secondary to an effect on pumped ion transport.

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Motor Deficits after Transsection of a Bulbar Pyramid in the Cat

By

ARNE MOSFELDT LAURSEN and MARIO WIESENDANGER

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Abstract

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on the operated and unoperated side. Placing reactions were delayed or absent in contralateral limbs; struggling was diminished and there was a tendency for the paws to remain in awkward positions. After one month operated cats could be distinguished from unoperated cats only by diminished flexor reflexes in contralateral legs and by a slight delay in contact placing. Damage of the medial lemniscus produced the tendency to place contralateral paws in awkward positions lasting for one week. Unlike pyramidal section, lemniscal damage did not produce deficient flexor reflexes.

There is agreement that pyramidotomy in cats produces motor dysfunction in the contralateral limbs which can be described roughly as abnormalities in gait and posture, in placing and hopping reactions, and as a tendency to extension of the limbs, most clearly demonstrated when the animal is suspended in a sling with the legs hanging freely. The signs are described fully and the literature reviewed by Marshall (1934), Tower (1935) and Liddell and Phillips (1944). There is disagreement as to the extent, duration and cause of the motor signs. In Tower's animals the impairment was evident in such simple acts as struggling, clawing, scratching and walking and seemed to be permanent with no sign of further recovery after about one month. Marshall's animals survived up to 4 weeks; the degree of recovery in the various symptoms was different in each animal, but no constant set of symptoms was left at the end of the observation period.

Liddell and Phillips' animals survived up to one year and showed persistent increased resistance to pressure to the pad of the contralateral limbs, measured with a dynamometer, as well as inability to walk on a ladder or pipe, the affected limbs

TABLE I Motor deficits in extremities contralateral to pyramidotomy in 13 cats

Cat	Survival period in weeks	Flexor posture of pendant limbs	Struggling	Awkward positions of paws	Gait	Visual placing	Contact placing
A 1	4	Reduced	Reduced*	No	Normal	Delayed*	Delayed
A 2	8	Reduced	Reduced*	Yes*	Slipping*	Normal	Delayed
A 3	1	Reduced	Reduced*	No	Normal	Delayed*	Delayed
A 4	2	Reduced	Reduced*	Yes*	Slipping*	Delayed*	Absent
B 1	5	Reduced	Reduced*	Yes*	Dragging*	Delayed*	Delayed
B 2	10	Reduced	Reduced*	Yes*	Normal	Delayed*	Delayed
B 3	20	Reduced	Reduced*	Yes*	Slipping*	Delayed*	Delayed
B 4	7	Reduced	Reduced*	Yes*	Dragging*	Delayed*	Delayed
C 1	4	Reduced	Reduced*	No	Normal	Delayed*	Delayed
C 2	2	Reduced	Reduced*	No	Normal	Delayed*	Delayed
C 3	14	Reduced	Reduced*	No	Slipping*	Normal	Delayed
C 4	5	Normal	Normal	No	Normal	Normal	Normal
C 5	4	Reduced	Reduced*		Normal	Normal	Delayed

* Returning to normal after one week

slipping off and remaining helplessly protruded downwards. Tower considered the fundamental loss of function to be a loss of phasic activity, chiefly flexor, whereas Ranson (1932) and Liddell and Phillips (1944) ascribed their findings to extensor hypertonus.

Tower attributed the greater severity and the permanence of the motor defect in her animals to the fact that the pyramidotomies in her series were complete whereas some of Marshall's were incomplete. Involvement of the medial lemniscus was a feature of the lesion in both series and was described as massive in Tower's series. Liddell and Phillips (1944) thought absence of hypertonia could be due to damage or oedema involving the inferior olive or the medial fillet.

We have investigated cats with complete and incomplete section of one bulbar pyramid and cats with combined lesions of one pyramid and of the medial lemniscus in the medulla oblongata, animals with stereotactic lesions of the medial lemniscus in the mesencephalon served as controls. Slow motion cinematography and recording of muscle action potentials were used to analyze movements and posture.

Material and Methods

Surgical procedures. Experiments were made on 16 cats 18–25 kg. operated under pentobarbital sodium anaesthesia. In 13 cats the pyramid was exposed by a ventral approach. Schiff (1939), Liddell and Phillips (1944) was viewed through an operating microscope and cut with a knife at the level of the trapezoid body. Incision of the dura often produced bleeding from tiny dural vessels which was stopped by compressing the vessel with a fine forceps for 2 min. No other bleeding was encountered. All animals were treated with terramycin until they were afebrile for 3–7 days. In 3 cats an electrolytic lesion (2.5 mA D.C. 20 sec.) was placed within the mesencephalic portion of the

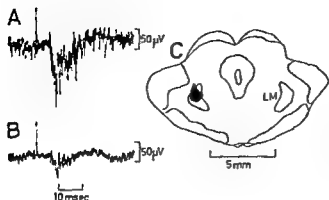


Fig. 1 Stereotactic lesion in the medial lemniscus. Correct placement of the electrode in the mesencephalon was ascertained by recording the response evoked by electrical stimulation of the skin of the contralateral paw (A). The response was reduced (B) after the lesion indicated by the black area (C).

lemniscus as shown in the atlas of Jasper and Ajmone-Marsan (1960). A needle with a 1 mm bared tip was inserted to coordinates 4 mm frontal, 5 mm lateral and 3 mm vertical. In one animal the vertical coordinate was ascertained by recording the gross response evoked by electrical stimuli delivered to the contralateral forepaw (Tarnecki 1962). The response had a latency of 6 msec and followed the stimuli at 10 sec. The amplitude of the response was 150 μ V (Fig. 1 A) before and about 50 μ V (Fig. 1 B) after the lesion was made. The site and extent of the lesions were verified histologically.

Testing. Posture and movements of the freely moving cats were studied by photography and slow motion cinematography soon after recovery from anesthesia and once a week for one to 20 weeks after the operation (Table 1). Flexor reflexes were elicited by strong stimuli pinching the pad, or by weak stimuli gentle stroking or by directing a jet of air on the paw. The scratch reflex was elicited either as an unconditioned response to scratching of the skin around the ear or as an instrumental response to obtain food (Jankowska 1959).

To record muscle action potentials the cats were suspended in a sling with the extremities hanging freely.

Initially the sling position is more disturbing to a cat than the position preferred by Liddell and Phillips (1944) with the rump in the observer's lap, the hind limbs unsupported and the spine vertical. This position, however, elicits tonic extension of both hind legs in normal cats. Tonic extension of the legs, struggling or signs of anxiety were absent in cats trained in the sling for an hour each day for about a week. The recordings were taken without and often with light sedation (10 mg/kg pentobarbital sodium). Trains of electrical stimuli were applied through needle electrodes to the tibial nerve at the ankle at intervals of 5 sec (5 pulses of 0.1 msec duration at 250 sec). The stimuli were 10 000 and 30 cycles/sec. The activity was recorded continuously on film moving at 2.5 mm/sec from the face of a two beam oscilloscope: the stimulating current was recorded on one beam as the voltage over a 100 ohm resistor in series with the stimulating electrodes.

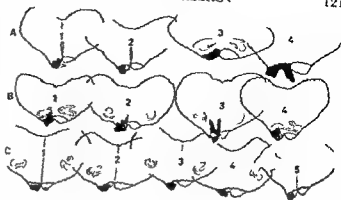
Histology. The cats were perfused under pentobarbital sodium anesthesia with 10% formalin in Ringer's solution. Serial sections 20 μ from the region of the lesion were stained by the Spilmeyer or Weil technique. Sections from above and below the lesion in one animal (B, Fig. 2) were stained by the Marchi technique.

Results

Neurological examination

1 Cats with unilateral pyramidotomy. Motor deficits involved the contralateral limb and were not systematically different when the lesion destroyed one bulbospinal pyramid cleanly (A, Fig. 2) or was incomplete in one pyramid (C, Fig. 1). The signs gradually diminished and after one month the only abnormalities were weak flexor reflexes in contralateral legs and a slight delay in contact placing. Cats with lesions involving

Fig 2 *Lateral pyramidal lesions in medulla oblongata.* Absent or destroyed tissue is indicated in black. In four cats (A) the lesions were complete without damage to other structures. In 4 cats (B) the lesions were too large involving in addition the medial lemniscus and reticular formation. In 5 cats (C) the pyramidal lesions were complete.



the medial lemniscus and reticular formation as well as one pyramid (B, Fig 1) differed only with respect to the position of the paws at rest and during walking (see p 6).

Movements. When struggling the cats used the affected limbs less often and less forcibly than the normal limbs. When dropped from table height the cat usually regained its position normally, evidencing weakness or slipping of the hind leg on the affected side for no more than the first few postoperative days. When grooming, clawing, playing, feeding and fighting, however, they used the affected and normal paws alike. One cat was trained for another purpose to obtain food by pressing a lever and chose to do it with the right paw before as well as after left pyramidotomy.

Gait. Stepping movements were alike on the two sides in seven cats. Six cats showed slight abnormalities in gait: the affected paws were not lifted from the floor during the flexion phase of the step and they sometimes slipped sideways. Slipping was especially frequent when the cat walked along a narrow ledge or a ladder (Marshall 1934) and the limb was then not replaced quickly or accurately. This sign lasted one week after the operation.

Posture. The posture was usually normal both in the sitting and standing positions. When the lesion involved the medial lemniscus the resting position of the affected limbs was sometimes awkward (illustrated in 1 Fig 3). This sign lasted one week after the operation.

Placing reactions. Visual placing was defective for the first postoperative days: the paw contralateral to pyramidotomy was not placed or was placed later than the normal paw, even when the cat could see the edge of the table (illustrated in 5 and 6 Fig 3). Defective contact placing was still present as long as the animals were followed (survival time given in Table 1) when their eyes were shielded the cats placed the affected paws with a delay.

Extensor tone and flexor activity. When the cat was suspended in a sling with the legs pendant the affected limbs hung in the extended position whereas the normal limbs were flexed (illustrated in 4 Fig 3 and in Fig 4) the difference between the sides being evident as long as the cats survived. Table 1. Ther

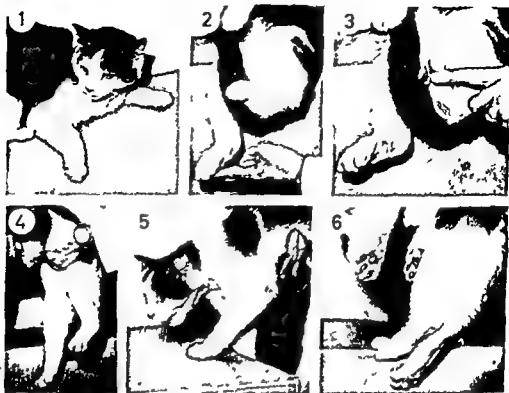
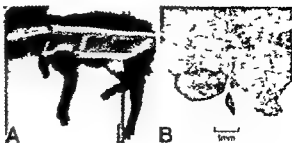


Fig. 3. Cat with pyramidotomy on the left side also involving the medial lemniscus (group D, Fig. 2) photographed 4 days postoperatively. 1. Awkward position of the right forepaw. 2. A flexion reflex in the right hind limb was not elicited by touch. 3. Normal flexion reflex in the left hind limb. 4. Cat lifted off the ground, note extended position of right hind limb. 5. and 6. Absence of visual placing reactions in right extremities.

in resistance to passive stretch of the limbs on the two sides. Supporting a paw yielded about the same resistance on both sides, except that the normal leg was more often withdrawn when the cat struggled. A difference in resistance to passive flexion was sometimes felt when the cat was apprehensive; the difference disappeared, however, when the cat was further accustomed to the sling position, to the observer, and to pressure applied to the pad. Flexor reflexes, whether elicited by strong or weak stimuli, were weaker on the side contralateral to pyramidotomy, or they were absent. The difference between the two sides was most pronounced when the stimuli were weak (illustrated in 2 and 3, Fig. 3), it diminished gradually but lasted throughout the survival period.

Scratch reflex. Flus reflex could be elicited with about the same threshold on both sides after pyramidotomy. The initial movement of the scratch reflex, reinforced by food after conditioning (p. 119), was performed equally well before and three weeks after pyramidotomy. 3 cats of group A.

Description of the lesions. Fig. 2. Group A. Four cats had complete unilateral lesions of the pyramids without damage to lemniscal or reticular fibres.



SPONTANEOUS ACTIVITY

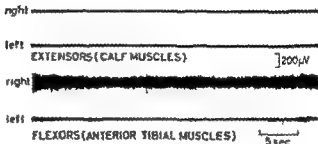


Fig 4 Extended left limbs in an alert cat (4) 7 days after pyramidectomy (B) on the right side (H&E stain). Action potentials were absent in extensor muscles. The affected limbs hang low because flexor tonus normally elicited in this position (right side) was reduced.

Group B In five cats the unilateral lesions were too large, involving part of the medial fillet and part of the reticular formation in addition to pyramidal fibres. Ascending degeneration in the medial lemniscus was evident in the Marchi stained sections of cat B 2.

Group C In four cats the lesions were too small, involving only a part of one pyramidal tract, other structures were not damaged. Cat 1 had a small lesion of the contralateral pyramid.

II Cats with unilateral medial lemniscotomy. The lesions interrupted at least half of the fibres in each of the three cats (Fig 1 C). Complete interruption of the medial lemniscus was not attempted because it would involve a large part of the reticular formation. Neurological deficits were small and lasted no more than one week. A backward position of the extremities contralateral to the lesion were observed in all cats during stepping the affected limbs sometimes slipped on the floor. There was a slight delay in the contact placing reaction. When the cat was lifted from the ground the extremities of both sides struggled equally and had the same tendency to end in a flexed position. The flexion reflex was the same on the two sides with both strong and weak stimuli.

Muscle action potentials

1 Cats with unilateral pyramidectomy. When the cat was suspended in a sling with the legs hanging freely, activity in extensor muscles was absent on both sides and activity was present in the flexors on the normal side (Fig 4). That the limbs contralat

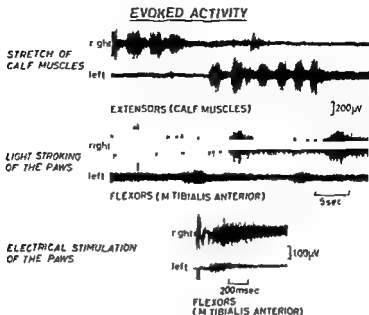


Fig. 5. Action potentials recorded from flexors and extensors of the hind limbs 7 days after section of the right pyramidal tract in a cat. *Extensors* (calf muscles) and *flexors* (M. tibialis anterior) are shown. The right limb is ipsilateral to pyramidotomy.

to pyramidotomy, hung lower than the normal legs was therefore due to decreased tonic flexor activity (Fig. 4). Electrical activity induced by stretch was about the same in the calf muscles on both sides. Light stroking of the dorsum of the paw or air jets directed at the paw elicited more muscle activity in the anterior tibial muscle of the ipsilateral than of the contralateral limb. Similarly, trains of electrical stimuli of two times threshold strength applied close to the ankle nerves of the two sides elicited briefer and smaller bursts of action potentials on the contralateral than on the normal ipsilateral side. The difference in response on the two sides was most pronounced during light sedation (Fig. 5).

II. Cats with unilateral medial lemniscotomy

When the cat was suspended in a sling with the legs pendant, flexor activity was equal on the two sides and extensor activity was absent. Air jets directed at the paws elicited the same amount of activity in the anterior tibial muscles of the two sides.

Discussion

The motor deficits produced by unilateral pyramidotomy in our cats corresponded closely to Marshall's (1934) description though the degree of involvement seems to have been less and recovery even faster in our animals than in his, and all the motor signs were contralateral. Initially the gait could be abnormal, the legs not being

lifted normally or slipping sideways. Awkward positions of the paws could be uncorrected, the animals could fall to the affected side when pushed from a table and the hind leg could then slip sideways. By confining lesions to the medial lemniscus we were able to show that these motor deficits were due to the lemniscal damage which often accompanies pyramidal section at the bulbar level and not to the pyramidotomy as such. These signs lasted a few days to a week.

Of the signs due to pyramidotomy, less use of the affected limbs in struggling, difficulty in walking on a ladder and absent or delayed visual placing recovered within a week. In agreement with Liddell and Phillips (1944), delayed contact placing and the extended position of the pendant legs were persistent for the survival period in our animals up to 5 months and in theirs to 12 months.

At no time did we see the inertia in initiating phasic movements such as clawing or scratching which was like the other motor signs permanent in Tower's (1933) animals. Indeed in our animals the scratch reflex to conditioned and unconditioned stimuli was elicited and executed normally on the two sides. We are at a loss to explain the discrepancy between our Marshall's (1934) and Liddell and Phillips (1944) findings on the one hand and those of Tower (1933) on the other. The only explanation we can offer is that her lesions were larger. Some of our sections, though dividing the pyramid completely, were confined to it and our lesions placed in the medial lemniscus destroyed no more than half the fibres. She describes her pyramidal lesions as being complete and as involving massive homolateral degeneration of the medial lemniscus.

We cannot agree with Liddell and Phillips (1944) that extensor hypertonia accounts for the extended position of the pendant limbs. They measured the pressure which had to be applied to the pad to cause the leg to flex since it was consistently higher contralateral to pyramidotomy (sometimes exceeding the body weight of the animal), they concluded that extensor tone was increased by pyramidotomy. We interpret the difference they found as due to a normal flexion reflex on the ipsilateral side elicited on the background of tonic extension of both hindlegs normally present in cats held as described by Liddell and Phillips (1944) (p. 120). By recording the muscle action potentials from flexors and extensors of the leg we were able to confirm Tower's conclusion based on her observation of the motor deficits that the primary disorder after pyramidotomy involves flexion. We found that the extended position of the pendant leg is due to inactivity of the flexors and that the flexors activated electrically through the spinal reflex pathway responded less briskly on the affected than on the normal side. The activity recorded in the extensors in response to stretch was the same on the two sides. This effect of pyramidotomy is in agreement with stimulation experiments: pyramidal volleys elicited flexion movements and inhibited extensor activity (Kato, Takamura and Fujimori 1964; Laursen and Wiesen 1966).

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Adrenergic Nerves in Spinal Ganglia of the Cat

By

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Adrenergic nerve fibres are present in the feline Gasserian ganglion in which they are related to both the ganglion cells and blood-vessels (Santini 1966). These findings prompted similar studies on other sensory ganglia, on which investigations are in progress. The present report gives preliminary data showing that adrenergic nerves occur in spinal ganglia of the cat.

Five cats were killed, and spinal ganglia from the cervical, thoracic, lumbar, and sacral levels were dissected out bilaterally. They were treated according to the method of Falck and Hillarp for the fluorescence microscopic visualization of adrenergic nerves (see Falck and Owman 1965). Under the conditions used the catecholamines are transformed into fluorophores emitting an intense green light.

Nerve cells with a specific amine fluorescence were not found in the ganglia. The cells varied considerably in size, and contained a varying amount of yellow-brown autofluorescent granules in the cytoplasm (Fig. 1).

Immediately outside the ganglion, bundles of green fluorescent axons of both the smooth and varicose type were seen in the fascicles of myelinated nerves. At the ganglion border the fluorescent bundles split up into smaller branches. Delicate varicose terminals, having the fluorescence characteristics of primary catecholamines, coursed isolated in various directions among the myelinated nerves. Many, if not all, of these adrenergic fibres could be directly related to blood vessels.

In the thoracic ganglia a moderate number of varicose nerve terminals, often single, coursed among the ganglion cells (Fig. 1). Some fibres ran contiguous to and encircled the perikarya — usually those of larger size — in a manner suggestive of a synaptic arrangement (Fig. 1). Other nerve fibres enclosed small blood vessels. Several isolated varicose axons could be followed for a considerable length in between the ganglion cells. The number of intraganglionic adrenergic nerve terminals was largest in the thoracic ganglia. Fewer nerves were present at the cervical level and only scattered fibres occurred in the lumbar and sacral ganglia. A relation of adrenergic nerve terminals with non fluorescent ganglion cells could be demonstrated at all levels studied.

The origin of the adrenergic fibres in these ganglia is as yet unknown. They may arise from the sympathetic chain. Cervical sympathectomy causes a severe reduction in the number of adrenergic nerve terminals in the thoracic ganglia.

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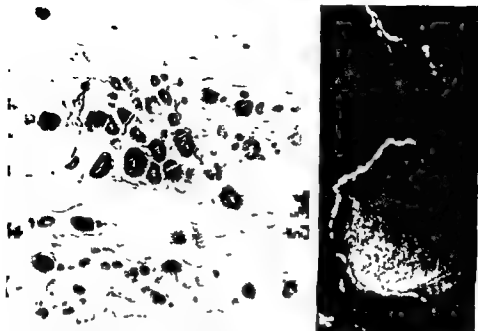


Fig. 1. Fluorescence micrograph of spinal ganglion from the thoracic level. All fluorescence within perikarya is autofluorescence. Left: Survey picture. Green fluorescent nerve terminals run between ganglion cells and in bundles of myelinated axons. 70 \times . Right: Varicose adrenergic nerve terminal encircling large ganglion cell in a synaptic manner. 400 \times .

in the amount of green-fluorescent fibres within the Gasserian ganglion of the cat (Kågström, Owman and Santini, unpublished observations). The possibility of a central origin should also be considered (cf Mitchell 1953, Dahlström and Luxe 1965). Experiments are in progress to obtain information on this problem.

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The Effect of Cold Exposure on the Catecholamine Excretion of Rats Treated with Decaborane

By

D G JOHNSON¹

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Abstract

JOHNSON, D G *The effect of cold exposure on the catecholamine excretion of rats treated with decaborane* Acta physiol scand 1966 68 129—133

Rats treated with decaborane exhibited a marked reduction in the noradrenaline (NA) content of heart, liver and spleen but no significant fall in adrenal catecholamines. Decaborane treated animals excreted less NA but more adrenaline (A) than control animals. When exposed to 3° C control animals survived and showed increased NA excretion in contrast to decaborane treated rats which however responded with increased excretion of A until death after 4—6 days. Unlike adrenalectomized controls adrenalectomized rats treated with decaborane failed to increase their NA excretion and died after 4—6 days.

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Studies of urinary excretion of catecholamines in man and animals exposed to cold (Arnett and Watts 1960, Cottle 1960, LeBlanc and Nadeau 1961, Leduc 1961) have drawn attention to the important role of both the adrenergic nervous system and the adrenal glands in cold adaptation and survival. It has also been shown that catecholamine-depleting drugs such as reserpine prevent the normal physiological response to cold, leading to poor adaptation and death (Dandiya, Johnson and Sellers 1960, Hoffman 1959, Zilberstein 1960, Taylor 1961). The failing NA excretion on exposure to cold also suggests inhibition of NA synthesis by reserpine (Leduc 1961, Johnson 1963).

Decaborane has been found to deplete both the brain (Merritt, Schultz and Wykes 1964) and peripheral organs (Euler and Lushajko 1965) of NA. The purpose of this study was to determine whether an increased adrenergic activity can be elicited when tissue catecholamines are depleted by decaborane. In addition it was thought that further evidence might be obtained for the role of the adrenergic nervous system and the adrenal medulla in cold adaptation.

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TABLE I Catecholamine content of organs of control rats and rats treated with decaborane. Mean \pm SEM

Organs	Control rats		Decaborane treated	
	NA	A	NA	A
Heart ng/g	350 \pm 28	82 \pm 38	477 \pm 31	75 \pm 19
Spleen ng/g	331 \pm 22	98 \pm 40	362 \pm 141	36 \pm 36
Liver ng/g	331 \pm 26	10 \pm 03	61 \pm 15	16 \pm 08
Adrenals μ g/kg BW	260 \pm 65	102 \pm 8	241 \pm 90	893 \pm 186

Methods

All experiments were carried out on male rats (300–350 g) of the Sprague-Dawley strain. The

funnels and a glass trap system for the separation of feces. The urine was adjusted to pH 3–4 by

was dissolved
12 mg/ml
succeeding
alternate days. Control animals received animal injections of 10 μ g decaborane daily. Organ deter-
minations were made after 6 days of this treatment.

Results

The doses of decaborane used in these experiments resulted in occasional mortality among rats kept at 20 °C (15% of the rats were dead at the end of 1 week of treatment). Adrenalectomy did not significantly affect the mortality after decaborane treatment. All of the rats receiving decaborane became lethargic and sedated, but could easily be aroused by handling. Rats treated with decaborane lost appetite and showed an average weight loss of 15 g per week.

Organ catecholamines. Decaborane caused a marked reduction in the NA content of heart, spleen, and liver. Table I. After 6 days of treatment with decaborane (3 injections) the NA content of the heart and spleen was less than 14% of control levels, while the liver contained less than 19% of control levels. The A content appeared to be little changed, except for an insignificant decrease in the spleen. The adrenal glands showed no decrease in NA content and only a slight insignificant decrease in A.

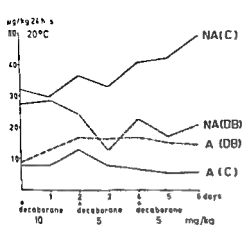


Fig 1

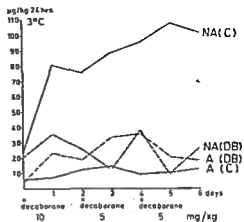
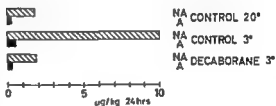


Fig 2

Fig 3 Effect of decaborane (10 mg/kg) on the urinary excretion of catecholamines from adrenalectomized rats exposed to 3°C. 6 rats per group. The decaborane treated animals died within 20 hrs.

CA EXCRETION IN URINE IN ADRENALECTOMIZED RATS (6 PER GROUP)



Urinary catecholamine excretion The urinary excretion of NA in rats treated with decaborane and placed at 20°C decreased over the course of 3 days to about one half of normal values (Fig 1). In the same time period the A excretion increased to approximately twice normal levels. The urinary levels of NA and A for control and decaborane-treated rats placed at 3°C are shown in Fig 2. The control rats increased their NA excretion in the first 24 hrs and maintained this for the duration of the experiment. The control A values increased to twice the value obtained at 20°C. The decaborane treated rats did not increase their NA excretion. The NA levels rose slightly the first day, followed by a decrease to levels below that which was normally excreted at room temperature. The A levels of decaborane treated animals rose markedly over the course of 4 days, whereafter the decaborane treated rats became moribund, and the urine volumes as well as the NA excretion tended to fall.

All of the control animals survived the exposure to 3° C, whereas none of the decaborane-treated animals lived more than 11 days.

Adrenalectomy. In the experiment with adrenalectomized rats at 3° C the control animals responded with a marked increase in NA levels similar to those of non-adrenalectomized rats (Fig. 3). The A excretion of the adrenalectomized controls rose to a level similar to that excreted by non-adrenalectomized rats at room temperature. Decaborane-treated animals failed to increase their NA excretion when placed at 3° C. Their excretion of A also failed to rise, and they died in less than 20 hrs. No adrenalectomized control animals died at 3° C.

Discussion

The decrease in tissue NA content after decaborane treatment agrees with the results obtained by other workers (Merritt *et al.* 1964, Euler and Lishajko 1965). The decrease to levels below 20 % in the liver, spleen, and heart compared to the decrease to 40 % observed in the brains of rats given similar doses of decaborane by Merritt *et al.* (1964) suggests some barrier to decaborane absorption in the brain or that the brain catecholamine-containing neurons are less sensitive to decaborane. The slight effect on the adrenal glands is also conspicuous.

The finding that NA excretion in urine only decreases to 50 % of control levels when the organ content of NA is decreased to much lower levels could be due to changes in the per cent of released NA which is excreted in the urine, or it could indicate that despite severe depletion the NA remaining in the adrenergic neurons is still available for a certain minimal level of activity. This observation agrees with the evidence obtained from simultaneous studies of urinary excretion and tissue content of catecholamines after reserpine (Ledue 1961). The rise in A excretion of rats treated with decaborane may reflect a direct releasing effect of decaborane on the adrenal gland.

The failure of the decaborane-treated rats to increase NA excretion in response to the cold stress suggests a diminished supply of transmitter, presumably owing to deficient biosynthesis of NA.

It is known that A is released in animals subjected to severe cold exposure (Hartman *et al.* 1923, Cannon *et al.* 1927, Saito 1928, Wadri, Seo and Abe 1935). However, as shown by later workers (Heroux 1955, Desmarais 1957, Ledue 1961), adrenalectomy does not prevent animals from adapting and surviving cold exposure. On the other hand Hoffman (1959) noted a complete loss of thermoregulation in adrenalectomized animals after treatment with reserpine and exposure to cold, with a certain degree of correction with A. However, Sellers *et al.* (1951) were unable to artificially acclimate clipped rats to cold with A. Because of the apparently slight effect of decaborane on the adrenomedullary stores of catecholamines found in this study, the use of decaborane seemed to provide an unusual opportunity to study the response of this organ to cold while at the same time selectively suppressing the adrenergic nervous system. The comparatively large increase in A excretion in the

¹ Addition to proof: Merritt and Schulz (1964) have recently produced evidence which indicates that decaborane selectively inhibits the decarboxylation of dopa.

urine of decaborane treated rats placed at 3° C and the rapidly occurring death of adrenalectomized animals treated with decaborane and exposed to cold seem to support the idea of A release as a functionally important physiological response to cold rather than just a coincident alteration in an animal unable to adapt. Assuming a recovery in the urine of 4 % of the A secreted by the adrenal glands (see Leduc 1961) the decaborane treated rats exposed to 3° C increased their daily secretion of A to amounts equivalent to the entire A content of the adrenal glands. This high release rate implies a considerable associated rate of synthesis, which can be taken as further evidence of the relatively slight effect of decaborane on the adrenal gland. The death of the decaborane treated rats exposed to cold after approximately 4—6 days suggests that A release provides only partial or temporary protection.

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Neurotransmitter Deficiency and Reloading in Noradrenaline Depleted Rabbits

By

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Abstract

BYGDEMÅN, S and U S VON EULER Neurotransmitter deficiency and reloading in noradrenaline depleted rabbits Acta physiol scand 1966 68 134—140

The vasoconstrictor effect of intermittent lumbar sympathetic stimulation of the hindleg of the rabbit pretreated with noradrenaline (NA) depleting doses of decaborane or prenylamine rapidly subsides in contrast to untreated controls. When the interval between the trains of stimuli is increased a partial recovery of the stimulation response is observed suggesting an accumulation of transmitter probably as a result of continuous resynthesis. Injection of NA (1—5 $\mu\text{g/kg}$) or dopamine (50—100 $\mu\text{g/kg}$) also causes an enhanced stimulus response suggesting uptake of NA and increased rate of synthesis respectively. An increase in stimulation response was observed after administration of dopa in prenylamine-treated but not in decaborane-depleted animals.

Under physiological conditions replenishment of the adrenergic neurotransmitter at the site of release occurs at such a high rate that no functional deficiency can be observed even during prolonged activity. In agreement with this no net loss of noradrenaline (NA) in the spleen of the cat was observed after stimulation of the splenic nerves for 10—15 min except in one animal in bad condition (Euler and Hellner Björkman 1955). More recently Dearnaley and Geffen (1966) have shown that on electric stimulation of the splenic nerve in cats for 1/2—5 min the neurotransmitter content in the stimulated part of the spleen decreased only by 7.7 per cent in comparison to an unstimulated part calculated on the DNA content of the tissue. However, after prolonged and intensive electrical stimulation of the sympathetic chain of the cat (Kernell and Sedvall 1961) succeeded in causing a considerable decrease of the NA stores in the gastrocnemius muscle.

After depletion of the NA stores in the cat with reserpine to such an extent that electrical stimulation of the lumbar sympathetic did not longer evoke vasoconstriction in the perfused leg it has been possible to resituate a vascular response by administration of NA or A. Burn and Rand 1958, Rosell and Sedvall 1961. This effect was of relatively short duration, indicating that the amount of NA taken up and available for transmitter purposes rapidly diminished during stimulation.

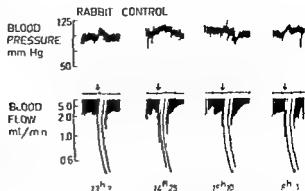


Fig 1 Control rabbit nembutal. Above systemic blood pressure below blood flow in hind leg. Arrows indicate start of 30 sec stimulation periods 20 sec followed by 2 min intervals. Time scale 1 min.

It therefore seemed of interest to study restitution of neurotransmission after depletion of NA by decaborane (Merritt, Schultz and Wykes 1964) and prenylamine (Segontin^R) (Schone and Lindner 1962).

It has recently been demonstrated that after depletion of the NA stores in the rabbit with prenylamine or decaborane, administration of NA or A is followed by an uptake into the stores to normal values (Mackenna 1965, Euler and Lishajko 1965 a). Moreover, it was found that refilling of the heart after depletion with prenylamine could be effected also by some NA precursors (tyrosine, dopa and dopamine) or to some extent even by the action of other amines (phenylethylamine, tyramine and octopamine) (Euler and Lishajko 1965 b). After decaborane depletion, dopamine but not dopa caused refilling of the heart (Euler and Lishajko, unpubl. obs.). It therefore seemed of interest to find out whether various precursors could restore transmission in a NA depleted system.

Methods

Rabbit 2-4 kg, male, and female, 10-12 weeks old, were used. The animals were anesthetized with nembutal 20-40 mg/kg i.v. or in some cases with urethane 1.5 g per kg i.v. The blood flow through the hind leg was measured by a Grass percutaneous catheter inserted into the femoral artery by a Grass percutaneous catheter inserted into the femoral vein.

The lumbar sympathetic chain was tied off at the level of the renal hilus, and bipolar platinum electrodes applied at the nearest distal interganglionic segment. Supramaximal stimuli of 10 msec duration 20 per sec were delivered for 30 or 60 sec with intervals of 1 or 3 min by means of an automatic device (Perman and Persson 1962). Untreated controls were prepared and stimulated in the same way.

Results

Untreated animals

In 3 animals the lumbar sympathetic was stimulated for 30 sec with 2 min interval for several hours. As shown in Fig. 1 the response is not perceptibly changed in the 4 stimulation periods between 13th and 16th hours.

RABBIT DECABORANE 6 mg/kg

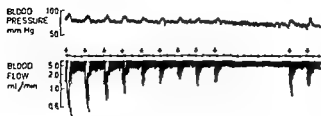


Fig 2 Rabbit decaborane 4 + 2 mg/kg 48 and 24 hrs previously. Declining vasoconstrictor response to 30 sec stimulation periods 20 sec intervals 2 min. Increased response after stimulus free interval. Time 1 min.

RABBIT DECABORANE 6 mg/kg

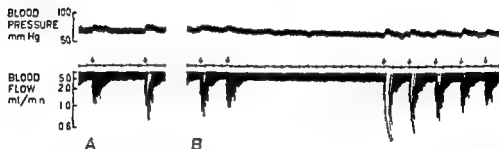


Fig 3 Rabbit decaborane 4 + 2 mg/kg 48 and 24 hrs previously. Increased response after exclusion of 1 (A) and 5 (B) stimulation periods of 60 sec 20/sec. Intervals 2 min. Time 1 min.

Decaborane depleted animals

The decaborane-treated animals had a characteristic sedated appearance with closed eyelids and few spontaneous movements. Their blood pressure was usually normal or slightly lower than normal at the beginning of the experiment with a tendency to fall gradually over the time course of the experiments.

The initial 30 sec nerve stimulations (600 stimuli) strongly reduced the blood flow through the leg as in the controls. However, in contrast to the untreated animal the vasoconstrictor effect gradually diminished in the decaborane treated animal. The rate of deterioration of the effect varied in different animals, presumably depending on the degree of decaborane action. Generally a definite decrease in response could be seen after 10–20 stimulation periods (Fig 2). If one or more stimulation periods were omitted, the response to the following stimulation period was markedly increased usually more so the longer the period of neuronal rest (Fig 2 and 3). This increase was of short duration and the pre rest response was again obtained during the 2nd to 4th post rest stimulation periods.

On repeated stimulation the decreasing response finally reached a certain low level which was maintained as long as stimulation periods and intervals were unaltered, as illustrated in Fig 2. If during this stage stimulation was continued for 1 min or longer the vasoconstrictor response gradually disappeared suggesting complete temporary depletion of the available stores. After a 1–3 min rest the response was restored to the same extent as before the previous stimulation.

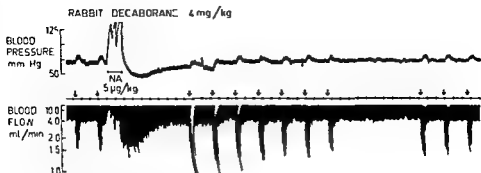


Fig 4 Rabbit, decaborane, 4 mg/kg 24 hrs previously. Increased response to 30 sec stimulation periods 20 sec, after NA 5 µg/kg i.v. Intervals 2 min. Time 1 min.

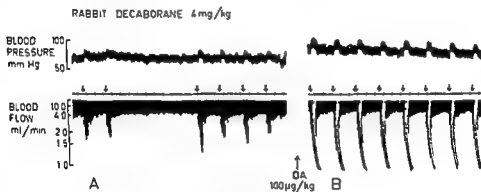


Fig 5 Rabbit, decaborane, 4 mg/kg i.p. 24 hrs previously. Increased stimulation response following neuronal rest (A) and 100 µg dopamine per kg i.v. (B). Stimulation periods 30 sec 20 sec. Intervals 2 min. Time 1 min.

Stimulation response after injection of NA

The effect of i.v. injection of NA was conspicuous (Fig 4). The smallest effective dose was 1–2 µg per kg. With doses of 5–10 µg/kg the effect was usually quite marked and lasted for 5–10 subsequent stimulation periods, indicating an uptake which could be utilized for neurotransmitter functions.

Stimulation response after injection of NA precursors and congeners

Dopamine in a dose of 100 µg/kg i.v. was found to produce a definite enhancement of the vasoconstrictor response to nerve stimulation (Fig 5), lasting for a considerable number of stimulation periods. Dopa or tyrosine, on the other hand, in a dose of 10 mg/kg was without noticeable effect, suggesting that these compounds, if taken up by the synthesizing apparatus, were not efficiently transformed to the transmitter under the prevailing conditions. No definite effect could be detected after isoprenaline 28 µg/kg, tyramine 0.5 mg/kg and octopamine 20 µg/kg.

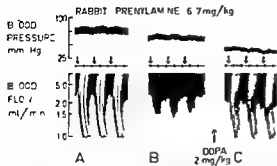


Fig 6 Rabbit prenylamine, 6.7 mg/kg *iv*. Stimulation period 60 sec 20 sec Intervals 1 min A Beginning of stimulation B After about 1 hr C 5 min after dopa 2 mg/kg *iv*

Prenylamine-depleted animals

Depletion after prenylamine given *iv* may be quite rapid as observed for the rabbit heart by Mackenna (1965). Thus the stores in the heart are largely depleted 1/2–1 hr after injection.

The vasoconstrictor response to lumbar sympathetic stimulation gradually diminished in a similar way as after decaborane when stimuli were applied 1/2 hr after the end of the infusion of prenylamine. The restoring effect of neuronal rest was also noted in the prenylamine-treated animals, although it was usually less marked than in the animals which had received decaborane.

NA 2–5 μ g/kg *iv* also restored the vasoconstrictor effect to some extent but did not restore the flow response. Dopamine 100 μ g/kg, although the effects were generally less conspicuous than in decaborane-treated animals. A marked effect on the stimulus response was observed even after dopa 2 mg/kg (Fig 6) in contrast to the observations in decaborane-treated animals.

Discussion

The experiments described in the present paper confirm in general the effects first noted by Burn and Rand (1958), and by Rosell and Sedvall (1961) in animals whose NA-stores had been depleted by reserpine. The same general features were observed in the decaborane and prenylamine-depleted rabbits which consisted of a gradual diminution of the response to vasoconstrictor stimulation and partial recovery after administration of NA.

In some respects our experiments have extended the observations made previously. Thus a partial recovery of the response was regularly seen after increasing the interval between the trains of stimuli. This effect suggests that the amount of transmitter available has increased during the prolonged interval.

Previous experiments have shown that exogenous NA is rapidly distributed between granular and extragranular pools in the decaborane-depleted heart (Euler and Lishajko 1965a). Thus redistribution between pools is less likely to be the underlying reason for the slow and gradual recovery on prolongation of the interval between stimulation. It appears more probable that both the weak residual effect on intermittent stimulation (Fig 2) and the partial recovery following "neuronal rest" depend on resynthesis occurring at a slow, approximately steady rate. The amount

of NA available for immediate use in stimulation would then be a function of the interval between two consecutive trains of stimuli. This interpretation does not, of course, exclude uptake as an auxiliary mechanism for the accumulation of transmitter. The recovery phenomenon was less marked in those animals in which the response to stimulation diminished rapidly, which was often the case in the prenylamine treated animals, suggesting a less efficient resynthesis.

It is of interest to note that in reserpine depleted animals Rosell and Sedvall (1962) did not observe spontaneous recovery after disappearance of the response to stimulation, even after prolonged stimulus free intervals. This seems to indicate that spontaneous resynthesis is impaired to a greater extent in their system. Infusion of moderate amounts of NA restored the stimulation response as did dopamine and dopa after reserpine.

No certain connection could be noted between height of blood pressure, blood flow and the appearance of the recovery after 'neuronal rest'. The importance of the flow for the vasoconstrictor response has been emphasized by Folkow (1962). The term 'neuronal rest' was used by Brown, Davies and Ferry (1961) in a somewhat different context and was intended to signify the long rest necessary for removal of transmitter from the receptor. In the present report the expression is used to represent the time interval necessary for accumulation of an extra amount of transmitter. This amount was small as suggested by its rapid disappearance following new trains of stimuli.

The partial recovery of the response after 'neuronal rest' had to be considered in the 'reloading' experiments with NA or its precursors, in which it was often necessary to increase the interval after the injection of the substances.

In view of the positive results on the recovery of the vasoconstrictor response observed by Rosell and Sedvall (1961) in reserpinized cats, it might be specially mentioned that dopa, in contrast to dopamine, was not effective in decaborane-treated animals, in doses up to 10 mg/kg.

According to Merritt and Schultz (1966) decaborane exerts a blocking effect on the decarboxylation of dopa. This might be a major contributing factor to the failure of dopa to restore the vasoconstrictor response in decaborane-depleted animals but not in reserpine treated ones. Dopa or tyrosine in doses of 50 mg/kg were also without effect on the NA content of the heart in decaborane-depleted rabbits (Euler and Lishajko, unpublished experiments), in contrast to prenylamine-depleted animals. As shown in the present study dopa was effective in restoring the vasoconstrictor response in prenylamine treated animals.

The experiments reported here give no information as to the relationships between neurotransmitter action and the amounts of transmitter present in the organ. However the depletion observed in various organs after decaborane or prenylamine makes it appear likely, that the transmission failure is due to depletion of the transmitter. The constant low level of response to stimulation in the depleted animals as well as the partial recovery after 'neuronal rest' supports the concept of a continuous slow resynthesis under these conditions. sufficient to

of transmitter during each stimulation period in depleted animals. The full response in the control animals speaks in favour of a rapid and efficient resynthesis which is in agreement with the findings of less than 10 per cent loss of transmitter in the organ after up to 5 min uninterrupted stimulation of the splenic nerves in the cat (Dearnaley and Geffen 1966). In the experiments of Euler and Hellner-Björkman (1955) it was observed that in one cat with low blood pressure and low initial NA values of the spleen 20 min nerve stimulation caused a 50 per cent fall in the NA content of the stimulated part. In this case the low control values may be indicative of a low synthesis rate.

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Intestinal Dipeptidases

Development of Dipeptidase Activity in the Small Intestine of the Rat as Related to the Development of the Intestinal Mucosa

By

TOR LINDBERG and CHRISTER ÖWMAN

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Abstract

LINDBERG, T. and ÖWMAN, C. *Intestinal dipeptidases. Development of dipeptidase activity in the small intestine of the rat as related to the development of the intestinal mucosa.* Acta physiol. scand. 1966, 68, 141-151.

The development of five intestinal dipeptidase activities (L-alanyl-L-glutamic acid, L-alanyl-L-proline, glycylglycine, glycyl-L-leucine and glycyl-L-alanine dipeptidase activity) in the rat from 15 days post coitum up to 3 weeks post partum was investigated and related to the structural development of the rat small intestinal mucosa. Very low enzyme activities were found in the period of mucosal cell proliferation (15 to 18 days post coitum). At 18 1/2 days post coitum the period of mucosal cell differentiation was commencing and simultaneously the dipeptidase activities began increasing to reach maximal values at parturition, by which time the differentiation was finishing. Within a few hours of birth when the newborns had suckled, an abrupt and significant fall to the relatively low adult level was observed for four of the dipeptidase activities. The fifth enzyme activity (L-alanyl-L-proline dipeptidase) declined more slowly postnatally and first reached the relatively low adult level during the 3rd week post partum. The relation of the changes in the intestinal dipeptidase activities to the structural characteristics of the mucosa during the development has been discussed.

In man the intestinal dipeptidase activities are present as early as 11 weeks of fetal age (Lindberg 1966 a). At this stage, the enzyme activities in the small intestine are of about the same magnitude as in adults (Lindberg 1966 b), and furthermore the mucosa of the small intestine in human fetuses is already rather well differentiated (Johnson 1910, Patzelt 1931). Therefore, it seems necessary to investigate intestines from still earlier stages of development. However, since adequate amounts of human fetal material could be obtained only at the period between 11 and 23 weeks of fetal age, an investigation was performed on developing rats. In the present report the dipeptidase activities of the small intestine were related to the structural development of the small intestinal mucosa at frequent intervals between 15 and 22 days post coitum. Further similar studies were performed at various postnatal stages up to 3 weeks post partum.

Material and Methods

Animals. A total of 252 fetuses were obtained from 35 albino rats weighing 150–200 g. In these

was assigned as day 1 *post coitum* in the study of rats used the length of the gestation period is 22 1/2 days *post coitum*. — At the appropriate stage the mother animal was decapitated under light ethyl ether anesthesia and the fetuses removed for enzyme studies and histological analysis. Those used for the enzyme studies were immediately put in a cold box (4°).

Newborn rats from 6 litters were taken immediately after delivery, before suckling. From 3 litters rats were also taken about 6 hrs after birth, these rats had thus sucked for a couple of hours.

part below the diaphragm in the older fetuses) at 5 μ in the sagittal plane. Staining was performed alternately in hematoxylin-eosin, McManus periodic acid Schiff (PAS) (Pearse 1960) and in Mallory's phosphotungstic acid hematoxylin (McManus and Mowry 1960). The preparations were mounted in Permount (Fischer).

in the cold (4°)

microscope. The length

etal age. In the younger

littermates were pooled

In the older fetuses (19–22 days *post coitum*) the small intestines were divided in two or three parts.

small intestine failed, however, and various amounts of colostrum was left.

In the older animals (from 3 days of life) the small intestine was slit longitudinally and the mucosa carefully cleaned by a dry soft cloth. The whole proximal, middle and distal 10 cm of the small intestine was taken and cut into pieces.

The separate preparations were homogenized in 1 ml (fetal material) or 2 ml 0.1 M NaCl in an icebath for 2 min at 14 500 rpm (MSL-homogenizer). After standing 30 min the homogenates were centrifuged (International model HR 1 27 000 g 30 min). After suitable dilution with redistilled water according to total nitrogen content the clear supernatants were used directly as the enzyme solution.

Assays. The dipeptidase activities were determined by the spectrophotometric method as previously described. The same method was used for the determination of the activities of the other enzymes.

Protein
(Amour

culated as 17 per cent of the protein content.

Unit of dipeptidase activity. One unit is defined as the activity hydrolyzing 1 μ mole of dipeptide per min at 40° (Jönsson and Lindberg 1963b).

Results

Development of small intestine

At the earliest stage analyzed (15 days *post coitum*) the stratified epithelium (Fig. 1A) lining the simple lumen of the intestinal anlage is about 30 μ thick, consisting of low columnar cells arranged in 2 to 3 layers. The oval nucleus occupies a considerable

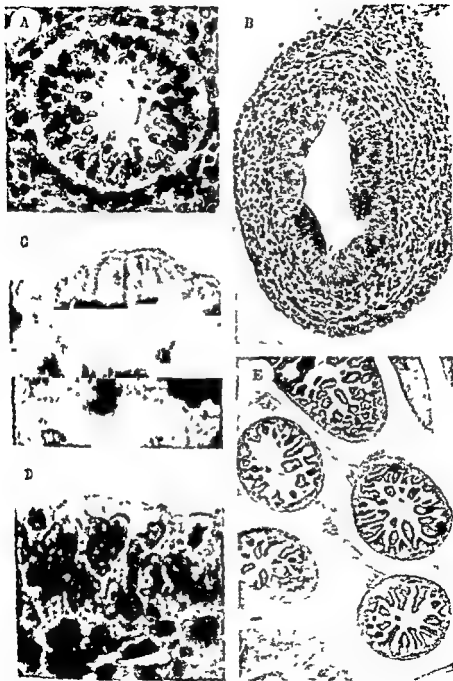


Fig. 1. Microphotographs demonstrating certain characteristics during development of rat small intestine.

(A) 15 days post coitum. Undifferentiated intestinal anlage with stratified epithelium. Hematec-x-van-eosin 420.

(B) 18 days post coitum. Early villi formation and intraepithelial vacuoles in small intestine. Intraepithelial vacuole in left upper part. Methyl-van-eosin 190.



Fig 1 cont

(C) 18 1/2 days *post coitum* Brush border at the surface of the simple columnar epithelium of jejunum Hematoxylin-eosin 1600 \times

(D) 18 1/2 days *post coitum* Brush border at the surface of the stratified epithelium of ileum Hematoxylin-eosin 1075 \times

(E) 20 days *post coitum* Survey picture showing well developed villi in jejunum Mallory stain mag. 40

(F) 22 days *post coitum* PAS positive inclusions in the supranuclear cytoplasm in one of the cells around a vacuole of cells in the distal small intestine Goblet cell (black) to the right PAS 1400

(G) 3 weeks *post partum* Entire supranuclear cytoplasm of columnar cells in distal ileum is occupied by a single large vacuole containing a lot of rounded PAS positive (black) inclusions PAS 240

portion of the cell. Large numbers of mitoses are collected along the luminal border. The thickness of the epithelium has increased by about 10 μ in the following stage (16 days) owing to increasing size of the columnar cells. At 17 days the cells of the 2 to 3 layers are arranged more irregularly. The thickness of the epithelium is again about 30 μ . Numerous mitoses are still present along the luminal border.

After this period of cell proliferation differentiation of the intestinal epithelium commences. Thus in the 18 day fetuses primitive villi begin to appear (Fig 1B). Proximally in the small intestine the epithelium forms longitudinally running ridges by thickenings consisting of up to 5 cell layers on top of a low mesenchymal core. Between the villus formations the stratified epithelium is only 2 layered. Intraepithelial vacuolization is a characteristic feature. Distally, the development is less advanced: the longitudinal ridges are barely recognizable and consists only of slight epithelial thickenings. Mitoses occur mainly within the thickened parts of the epithelium along the whole small intestine. At 18 1/2 days the ridges are more prominent in the distal portion of the small intestine due to the ingrowth of a low broad based mesenchymal core underneath the epithelium. Proximally the villi are well developed and covered by a simple columnar epithelium. At this stage a delicate brush border has formed at the luminal surface of the columnar cells.

throughout the small intestine (Fig 1C and D) In the next stage (19 days) no prominent change is seen in the appearance of the intestinal anlage

In fetuses 20 days old, prominent villi are found in the whole jejunum ileum (Fig 1E) The surface of the simple columnar epithelium is characteristically convex and the oval nucleus is quite large Mitoses are now relatively few and mainly collected in the intervillous parts Typical goblet cells have begun to appear within the epithelium and increase in number in the following stages

From the 21 day stage on the columnar cells of the epithelium are higher The luminal surface is flattened rather than convex, and covered by a well developed brush border The round nucleus is located in the middle or basal third of the cell Distally in the small intestine the supranuclear zone of the columnar cells often contain a small number of PAS positive granules of about $1\ \mu$ in diameter see further Owman 1963 1964 a and b) At 22 days *post partum* the intestinal epithelium has an appearance similar to that in the previous stage However the supranuclear portion of the cells in the distal ileum is as a rule occupied by a large vacuole and the PAS positive inclusions ($1-2\ \mu$ in diameter) are then often found around its periphery (Fig 1F) The first sign of the crypts of Lieberkuhn is seen at this stage as a narrow epithelial invagination into the underlying connective tissue between the villi

In the postnatal stages studied the development of the crypts of Lieberkuhn proceeds more rapidly in the proximal than in the distal small intestine At 3 weeks *post partum* both crypts and villi are well developed The mitotic figures present are mostly located in the crypts

In the specimens taken immediately after birth before sucking the simple columnar epithelium lining the villi of the ileum has an appearance similar to that in fetuses 22 days old The cellular inclusions have increased somewhat in number and tend to coalesce into small groups in the cleared supranuclear cytoplasm About 6 hrs later when sucking has begun the cell nucleus is pushed towards the base of the cell and the supranuclear cytoplasm especially near the nucleus now contains a large number of irregularly formed PAS positive inclusions of $1-3\ \mu$ or more in diameter During the next 2 days the amount and size of the inclusions increase considerably At 2 weeks after birth the whole supranuclear cytoplasm is occupied by a small number of very large PAS positive globules measuring up to $10-15\ \mu$ the nucleus is pressed basally At 3 weeks (Fig 1G) the general amount of inclusions now about $4\ \mu$ in diameter is smaller and instead the entire supranuclear cytoplasm is present as a single large vacuole that compresses the nucleus towards the cell base — Very little inclusion material is found in the proximal portion of the small intestine at these stages

Development of dipeptidase activity

The assays of the various dipeptidase activities were performed at the respective pH-optimum (see below) The glycylglycine dipeptidase activity was determined in the presence of 5 μ moles Co^{2+} ions (final concentration $2.9 \times 10^{-5}\text{M}$)



Fig 1 cont

(C) 18 1/2 days post coitum Brush border at the surface of the simple columnar epithelium of jejunum Hematoxylin-eosin 1600 \times

(D) 18 1/2 days post coitum Brush border at the surface of the stratified epithelium of ileum. Hematoxylin-eosin 1025

(E) 20 days post coitum Survey picture showing well developed villi in jejunum Mallory staining 40

(F) 22 days post coitum PAS positive inclusions in the supranuclear cytoplasm in one of the cells around a vacuole of cells in the distal small intestine. Goblet cell (black) to the right. PAS 1400 \times

(G) 3 weeks post partum Entire supranuclear cytoplasm of columnar cells in distal ileum is occupied by a single large vacuole containing a lot of rounded PAS-positive (black) inclusions. PAS 240

portion of the cell. Large numbers of mitoses are collected along the luminal border. The thickness of the epithelium has increased by about 10 μ in the following stage (16 days) owing to increasing size of the columnar cells. At 17 days the cells of the 2 to 3 layers are arranged more irregularly. The thickness of the epithelium is again about 30 μ . Numerous mitoses are still present along the luminal border.

After this period of cell proliferation, differentiation of the intestinal epithelium commences. Thus in the 18-day fetuses primitive villi begin to appear (Fig 1B). Proximally in the small intestine, the epithelium forms longitudinally running ridges by thickenings consisting of up to 5 cell layers on top of a low mesenchymal core. Between the villus formations the stratified epithelium is only 2 layered. Intraepithelial vacuolization is a characteristic feature. Distally, the development is less advanced: the longitudinal ridges are barely recognizable and consists only of slight epithelial thickenings. Mitoses occur mainly within the thickened parts of the epithelium along the whole small intestine. At 18 1/2 days the ridges are more prominent in the distal portion of the small intestine due to the ingrowth of a low, broad based mesenchymal core underneath the epithelium. Proximally, the villi are well developed and covered by a simple columnar epithelium. At this stage a delicate brush border has formed at the luminal surface of the columnar cells.

throughout the small intestine (Fig 1C and D) In the next stage (19 days) no prominent change is seen in the appearance of the intestinal anlage

In fetuses 20 days old, prominent villi are found in the whole jejunum ileum (Fig 1E) The surface of the simple columnar epithelium is characteristically convex, and the oval nucleus is quite large Mitoses are now relatively few and mainly collected in the intervillous parts Typical goblet cells have begun to appear within the epithelium and increase in number in the following stages

From the 21-day stage on, the columnar cells of the epithelium are higher The luminal surface is flattened rather than convex, and covered by a well developed brush border The round nucleus is located in the middle or basal third of the cell Distally in the small intestine, the supranuclear zone of the columnar cells often contain a small number of PAS positive granules of about $1\ \mu$ in diameter (see further Owman 1963, 1964 a and b) At 22 days *post partum* the intestinal epithelium has an appearance similar to that in the previous stage However, the supranuclear portion of the cells in the distal ileum is as a rule occupied by a large vacuole, and the PAS positive inclusions ($1-2\ \mu$ in diameter) are then often found around its periphery (Fig 1F) The first sign of the crypts of Lieberkuhn is seen at this stage as a narrow epithelial invagination into the underlying connective tissue between the villi

In the postnatal stages studied the development of the crypts of Lieberkuhn proceeds, more rapidly in the proximal than in the distal small intestine At 3 weeks *post partum* both crypts and villi are well developed The mitotic figures present are mostly located in the crypts

In the specimens taken immediately after birth, before sucking, the simple columnar epithelium lining the villi of the ileum has an appearance similar to that in fetuses 22 days old The cellular inclusions have increased somewhat in number and tend to coalesce into small groups in the cleared supranuclear cytoplasm About 6 hrs later when sucking has begun the cell nucleus is pushed towards the base of the cell and the supranuclear cytoplasm especially near the nucleus now contains a large number of irregularly formed, PAS positive inclusions of $1-3\ \mu$ or more in diameter During the next 2 days the amount and size of the inclusions increase considerably At 2 weeks after birth the whole supranuclear cytoplasm is occupied by a small number of very large PAS positive globules measuring up to $10 \times 6\ \mu$ the nucleus is pressed basally At 3 weeks (Fig 1G) the general amount of inclusions (now about $4\ \mu$ in diameter) is smaller and instead the entire supranuclear cytoplasm is present as a single large vacuole that compresses the nucleus towards the cell base — Very little inclusion material is found in the proximal portion of the small intestine at these stages

Development of dipeptidase activity

The assays of the various dipeptidase activities were performed at the respective pH-optimum (see below) The glycylglycine dipeptidase activity was determined in the presence of 5 μ moles *L*-histidine final concentration 2.0×10^{-3} M

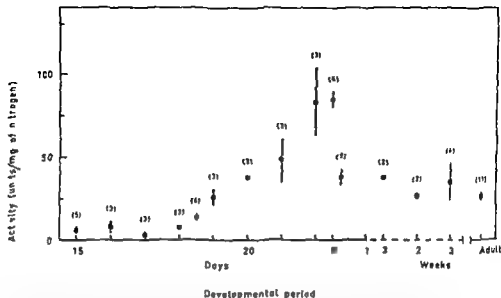


Fig 2 Pre- and postnatal development of glycyl-L-leucine dipeptidase activity (units per mg nitrogen mean values \pm S.E.M.) in the rat small intestine. The S.E.M. for 18 days post coitum is ± 0.4 for 20 days post coitum ± 0.9 and for 3 days post partum ± 0.3 (bars covered by symbols). Figures in parenthesis indicates number of litters. P - partus. Optimum pH and 4.0.

The results for glycyl-L-leucine dipeptidase activity are shown in Fig 2, in which the mean values and the standard error of the mean (S.E.M.) of the activity, expressed as units per mg nitrogen present in the enzyme solution, are given for the various litters from the different stages of the development. For the older fetuses as well as for the newborn rats and the sucklings, where various parts of the intestine were studied, the figures given are mean values calculated from the activities in the different intestinal portions. The values of the adult rats are taken from the previous investigation on the intestinal dipeptidases in the adult rat (Josefsson and Lindberg 1966).

It is apparent from the figure that glycyl-L-leucine dipeptidase activity is low between the 15 and 18 days post coitum. In the 18 1/2 day fetuses, however, the activity starts rising to reach a maximum around parturition. In animals allowed to suck the activity then abruptly falls already after some hours of life to the same relatively low level as that of the adults. The curves obtained for L-alanyl-L-glutamic acid, glycylglycine and glycyl-L-valine dipeptidase activity were similar to that for glycyl-L-leucine. However, the results for L-alanyl-L-proline dipeptidase activity differ (Fig 3). In this case the activity, which became maximal in those rats taken immediately after delivery, did not fall abruptly in the suckled newborns, but declined successively during the postnatal suckling period, to reach the level of adult rats first about 3 weeks post partum. Table 1 gives a survey of the various dipeptidase activities at certain characteristic stages of the development.

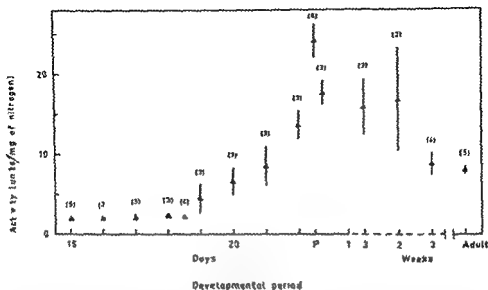


Fig. 3. Pre- and postnatal development of L-alanyl L-proline dipeptidase activity (units per mg nitrogen, mean values \pm S.E.M.) in the rat small intestine. The S.E.M. for 15 days post coitum is ± 0.14 for 16 days ± 0.24 for 18 days ± 0.18 and for 18 1/2 days ± 0.15 (bars covered by symbols). Figures in parenthesis indicates number of litters. P = partus. Optimum pH and 40

TABLE I. The pre- and postnatal development of the dipeptidase activities (units per mg nitrogen mean values \pm S.E.M. (n)) in the rat small intestine. Optimum pH. The assays of glycolylglycine dipeptidase activity was determined in the presence of 5 μ moles Co^{2+} ions (final concentration 2.95 $\times 10^{-4}$ M).

Developmental stage	L-Alanyl L-glutamic acid	L-Alanyl L-proline	Glycol glycine	Glycol L- leucine	Glycol L- valine
15 days post coitum	5.12 \pm 0.64 (4)	1.96 \pm 0.14 (3)	1.03 \pm 1.78 4	6.10 \pm 1.3 2	9.63 \pm 0.66 4
19 days post coitum	10.9 \pm 2.49 (3)	4.23 \pm 1.76 3	4.19 \pm 0.78 3	7.61 \pm 4.8 3	7.63 \pm 3.93 3
20 days post coitum	31.3 \pm 7.27 3	13.6 \pm 1.0 (3)	15.8 \pm 1.10 3	83.6 \pm 21.3 3	60.0 \pm 2.81 3
Newborn nonsuckled	26.4 \pm 3.3 6	24.1 \pm 7.0 6	12.4 \pm 3.8 1	84.3 \pm 7.3 1	87.3 \pm 3.36 1
Newborn suckled	10.9 \pm 0.81 3	1.7 \pm 1.4 3	14 \pm 1.8 3	5.9 \pm 4 3	40.0 \pm 4.95 3
3 weeks post partum	11.4 \pm 3.73 4	8.40 \pm 1.1 4	2.86 \pm 1 4	1 1	3.11 \pm 4.1 1
Adult	10.8 \pm 1.1 10	3 10	4 1	1 1	1 10

* From Josefsson and Lindberg, 1962

According to the Students *t* test the values of the non suckled and suckled newborns differ significantly for the activities of L-alanyl L glutamic acid ($p < 0.05$) glycylglycine ($p < 0.05$), glycyl L-leucine ($p < 0.001$) and glycyl L valine ($p < 0.001$). For L-alanyl L proline dipeptidase activity the difference is not significant ($0.1 > p > 0.05$).

The regional distribution of the dipeptidase activities along the small intestine was studied from 19 days *post partum*. The activities for L-alanyl L glutamic acid glycylglycine glycyl L-leucine and glycyl L valine were in all stages higher in the distal portions of the small intestine as compared to the proximal part. These findings were more marked in intestines from the 21 day stage up to and including the newborn period. On the other hand L-alanyl L proline dipeptidase activity was equally high in the various portions of the intestine in the prenatal period. In the following postnatal stages all the activities were slightly higher in the middle and distal sections of the gut thus resembling the conditions found in the adult rat intestine (Josefsson and Lindberg 1966).

Influence of pH and metal ions

The effect of pH and bivalent metal ions on the intestinal dipeptidases was investigated in fetuses aged 19, 20 and 21 days *post partum*. Various phosphate and borate buffer solutions were used to obtain a pH range of 6.0 to 8.0 in the digest mixtures. The pH optima found for the various activities were as follows (pH optima for adult rat intestinal dipeptidases (Josefsson and Lindberg 1966) are given in parenthesis) for L-alanyl L glutamic acid dipeptidase 7.2 (7.3) for L-alanyl L proline dipeptidase 7.0 (7.0) for glycylglycine dipeptidase 7.5 (7.5) for glycyl L-leucine dipeptidase 7.4 (7.5) and for glycyl L valine dipeptidase 7.5 (7.4).

The influence of four bivalent metal ions (Co^{2+} , Mn^{2+} , Mg^{2+} and Zn^{2+} ions) when added in the concentration of 10 μmoles (final concentration $5.9 \times 10^{-4}\text{M}$) on the various dipeptidase activities was essentially the same as that found in the adult rat (Josefsson and Lindberg 1966). However a difference was found for the glycyl L valine dipeptidase activity which was reduced by Co^{2+} ions by about 30% in the fetuses while these ions were without influence on this activity of the adult rat.

Discussion

The present results on the development of the small intestine (jejunum ileum) demonstrate that after a period of cell proliferation in the striated epithelium the mucosa shows a rapid differentiation from the stage of 18 days *post partum* at parturition 4 days later the villi appear well developed. At 18 1/2 days *post partum* the epithelium in the portion of the small intestine is of a simple columnar type. A delicate brush border can be observed at this stage throughout the small intestinal anlage. Typical goblet cells are found at 20 days. The first sign of crypt formation occurs at 22 days *post partum*. In principle the present findings agree with those

earlier reported (Kammeraad 1942). In the specimens taken 3 weeks after birth both the crypts of Lieberkuhn and the villi have essentially reached final development.

From 21 days *post partum* the epithelial cells, especially in the distal small intestine, contain PAS-positive inclusions, which probably are of muco- or glycoprotein nature (Owman 1963). The inclusions may be associated with the perinatal transmission of immune proteins occurring *via* the gut (Halliday 1955 a, Brambell and Halliday 1956, von Mayersbach 1958, Clark 1959, Owman 1964 b). By this route the rat offspring achieves most of its passive immunity from the mother up to 3 weeks postnatally (Halliday 1955 b and 1956).

When relating the structural development of the mucosa to that of the intestinal dipeptidase activities, certain observations should be pointed out. Thus it is evident that the increase of the enzyme activities (expressed as units per mg nitrogen) from the low level in the cell proliferation period coincides with the differentiating period of the mucosa. It is also apparent that the activities reach maximal values at the end of the latter period. Structural changes in the mucosal cells related to this increase are difficult to establish. The anlage of the crypts of Lieberkuhn are not seen until 22 days *post partum*, at which stage the activities already are maximal or almost maximal. Goblet cells of typical appearance are not present until the 20 days stage. It may be of interest that the brush border membrane appears simultaneously along the whole length of the small intestine at 18 ± 2 days, the stage when the enzyme activities begin to increase.

Larger amounts of the activities, except of L-alanyl L-proline dipeptidase activity, is found in the middle and distal thirds of the intestine. It should be noted that in these portions of the small intestine immune protein is absorbed perinatally (see above), and the first structural sign of this uptake is observed in the form of PAS-positive granules at the 21-day stage.

The newborn period requires special attention. The abrupt fall of the activities, except of L-alanyl L-proline dipeptidase activity, in the suckled newborns is remarkable. The decrease, which was observed in all the three portions of the intestine, but tended to be more pronounced in the proximal two thirds, coincides with the appearance of increased amount of PAS positive material both in the intestinal lumen and within the supranuclear cytoplasm in the mucosal cells. This material is probably derived from colostrum, unlike the PAS positive granules found prenatally, which arise from the absorption of amniotic fluid (Owman 1964 b). Control experiments Lindberg unpublished observation revealed that colostrum obtained from lactating rats has an inhibitory influence on the enzyme activities in intestinal extracts from fetuses aged 22 days as well as from adults. This inhibitory effect was less pronounced for L-alanyl L-proline dipeptidase activity than for the other activities, a finding which may explain the slow decrease of this activity in the suckling period. The nature of this inhibition of colostrum is under investigation. It should be recalled that the rat is a *polytrophic* species which acquire passive immunity by colostrum in the suckling period (Halliday 1955 a). Besides the possible effect of colostrum other factors such as bacterial invasion and

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Acid-Base Changes in Rat Brain Tissue during Acute Respiratory Acidosis and Baseosis

By

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Abstract

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The CO_2 buffer capacity of brain tissue *in vivo* was studied by measuring the tissue bicarbonate concentration at various tissue CO_2 tensions in rats which were either hyperventilated or breathed various CO_2 concentrations spontaneously for 30 min. Measurements of the tissue lactate content indicated that the technique of freezing the tissue *in situ* did not lead to tissue hypoxia. The main increase in the tissue bicarbonate concentration at a given inspired CO_2 concentration occurred within 30 min, but there was a slow further increase from 30 to 180 min. The values did not differ significantly between groups anesthetized with pentobarbital or phenobarbital and they were not

An adequate description of the acid base metabolism of a system involves the definition of any change in terms of respiratory and nonrespiratory ('metabolic') components. This has been done systematically for blood, although the best way of defining the respiratory and the nonrespiratory changes has yet to be agreed. Such changes have been derived either from *in vitro* titrations with various CO_2 concentrations, or from changes in the plasma when the organism is titrated with CO_2 *in vivo* (see discussion by Astrup, Siggaard Andersen, Relm and Schwartz in Symposium edited by Nahas 1966).

Apart from blood, comparatively little is known about acid base changes in tissues. The brain presents peculiar problems since it has a specialized extracellular fluid, the volume of which is difficult to measure, although it is possibly identical in composition to the cerebrospinal fluid (CSF). *In vitro* equilibrations of brain tissue homogenates with various CO_2 concentrations (Siecyo 1962 b, Kazemi and

Vithoefer 1964) will be of value for certain problems, but the procedure cannot give information on the buffer capacity of the intact tissue because extrapolations from such homogenates are of doubtful validity (Siesjö 1962 b), and the procedure disrupts the cell membranes, thereby abolishing the compartmentation of buffer groups. It is likely that many such groups in the intact tissue exist in compartments which have a pH very different from that in the homogenate, and that homogenization will affect the buffer capacity.

The present experiments were made to determine the *in vivo* buffer capacity of rat brain tissue. The work was made possible by the development of a sensitive and accurate method for the determination of the acid-labile CO_2 of tissues frozen *in situ* with liquid nitrogen (Ponten and Siesjö 1964 a, b), and by the determination of the CO_2 tension gradients in the tissue (Ponten and Siesjö 1966). The buffer capacity of the tissue agreed fairly well with that of whole blood *in vitro* although it was less than that obtained on cat brain tissue by an independent method (Roos 1965).

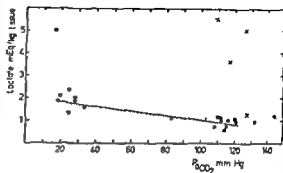
Methods

Experimental procedures. 126 male albino rats of the Sprague Dawley strain weighing 200–400 g were anesthetized with intraperitoneal pentobarbital or phenobarbital (60 and 100 mg per kg b.w. respectively). One femoral artery was cannulated for monitoring the blood pressure and for blood sampling. At each sampling the blood loss was 0.1–0.5 ml. The total blood loss during each experiment was 1–5 ml which was replaced with a balanced Krebs-Henseleit solution (Krebs 1950). The mean hemoglobin content of the blood fell from 15.9 ± 1.6 to 15.2 ± 1.4 g per cent (mean \pm S.D.) during the experiment. If the blood pressure fell below 80 mm Hg and if this could not be corrected within a few minutes the whole experiment was discarded. The animal was laid on a metal box which could be heated at intervals to prevent hypothermia and its head was secured in a head holder. At the end of the operation the skin over the skull was incised longitudinally to accommodate a paper funnel for freezing of the brain *in situ* (see below). The body temperature was measured in the rectum using a thermocouple electrothermometer.

When the operative procedures were finished the animal was left undisturbed for 10 min after which arterial blood was sampled. The animal then breathed air or CO_2 mixtures spontaneously or it was hyperventilated for a set period of 15 min to 3 hrs. The gas mixtures contained 0–18% CO_2 and 30–50% O_2 . The animals were hyperventilated with air or 10% O_2 using a miniature respiration pump (Palmer, London). During these periods one to three arterial samples were drawn. The last one immediately before the head of the rat was frozen. If the PaCO_2 of the two preceding samples differed by more than 10 per cent the experiment was discarded.

The technique of freezing the tissue *in situ* has been modified several times. Originally Ponten and Siesjö (1964 b) the whole rat was frozen by direct immersion into the liquid nitrogen. The preliminary account of the *in vivo* buffer capacity of the rat brain (Ponten 1964) was based on experiments in which only the heads were dipped into the liquid nitrogen. In the present experiments liquid nitrogen was poured onto the exposed craniotomies of the vertex through a paper funnel fitted into the skin incision. This technique made it possible to freeze the brain without further manipulation and pulmonary ventilation was not obstructed during the freezing. Provided the level of anesthesia was adequate there was only a slight stimulation of the respiratory system during freezing and spontaneous respiration resumed immediately after the thawing of the brain. The

Fig 1 Relation between the P_{aCO_2} and the lactate content of brains frozen *in situ* with different techniques. Rats, pentobarbital anaesthesia. The regression line refers to brains frozen by pouring liquid nitrogen onto the exposed frontoparietal bones (present method, filled circles). Crosses denote lactate values of brains frozen by immersing the whole head of the rat in liquid nitrogen. These brains had low T_{CO_2} values. Unfilled circles give the lactate values obtained from brains frozen with the same technique but giving high T_{CO_2} values comparable to those obtained with the present method.



joined with a glass stoppered side tube (cf. Siesjö and Thompson 1965). Both the main flasks and the side tubes has sockets used for perfusing the unit with CO_2 free nitrogen gas. With these modifications the coefficient of variation was still 0.9 per cent. The analyses were carried out on the supratentorial parts of the brain and thus the samples included ventricular as well as subarachnoidal cerebrospinal fluid.

The water content of the tissue was determined by drying pieces of tissue to constant weight at $105^\circ C$.

The bicarbonate concentration of the brain tissue (meq/kg) was calculated by subtracting the

derived value was the mean bicarbonate concentration of the combined water phase of the brain. This parameter was plotted against the mean tissue CO_2 tension to yield a CO_2 binding curve. In order to facilitate comparisons the equivalence of the pH term in a tissue system — $\log \frac{(HCO_3)}{P_{tCO_2} S_1}$ — was also calculated. In this ratio S_1 is equivalent to 0.0314 mmoles/g tissue H_2O/mm Hg (cf. Siesjö 1966, Siesjö and Pontén 1966b).

Results

Influence of the freezing technique on the total carbon dioxide and the lactate content of the tissue
The present study showed a higher tissue buffer capacity than that preliminary reported (Pontén 1964). This was due to a difference in the total CO_2 content of the tissue in hypercapnia, the new series giving the higher values. In hypocapnia, however, there was no significant difference in T_{CO_2} between the two sets of experi-

ments. In order to study whether the difference observed in hypercapnia was due to the modification of the freezing technique, control experiments were carried out with determination of total CO_2 and of lactate content in tissues from animals which were either exposed to 15 per cent CO_2 or hyperventilated for 30 min.

The modified freezing technique used in the present experiments led to consistent values for total CO_2 and lactate content. However, if the freezing technique of the previous series (Ponten 1964) was reproduced, both the lactate and the total CO_2 values were variable (Fig. 1). Thus, in all but one of the rats frozen with the previous technique and in which special care was taken to protect the airways from the liquid nitrogen, the T_{CO_2} values equalled those observed with the present freezing technique and the lactate content was normal. In 3 out of 5 similar experiments which gave low T_{CO_2} values the lactate content of the tissue was increased above the normal. The results suggest more than one reason for the observed differences. Obviously, the freezing technique previously used could cause a hypoxic condition as indicated by the increase in lactate but it was also found that the CO_2 of the gas mixture breathed by the rat could solidify if the liquid nitrogen made direct contact with the gas tubings. In such a case the CO_2 concentration of the inhaled gas mixture could fall to very low values, as verified by passing the gas through a CO_2 electrode.

The original freezing technique used in the laboratory (Ponten and Siesjö 1964 b) apparently interfered with the oxygenation of the animal *ante mortem* since it resulted in high tissue lactate values. Thus 14 rats anesthetized with pentobarbital were plunged into liquid nitrogen without previous treatment. The mean tissue lactate concentration in this group was 4.5 ± 0.4 meq/kg.

Comparison between pentobarbital and phenobarbital anesthesia. It has been reported (Schmahl 1965) that the redox quotient lactate/pyruvate in brain tissue will increase more from the normal value with pentobarbital than with phenobarbital. The latter drug is also associated with a higher ATP/ADP, and a higher phosphocreatine/creatine ratio in the tissue. Since all these substances should participate in the buffering of CO_2 in the tissue, a comparison was made between two groups of 17 and 40 rats, anesthetized with pentobarbital and phenobarbital, respectively. There was a slight difference in the plasma bicarbonate but not in the tissue bicarbonate concentrations between the two groups, phenobarbital giving a higher mean plasma standard bicarbonate (24.0 ± 0.4 meq/l, mean \pm SE) than pentobarbital (22.3 ± 0.8 meq/l) ($p < 0.05$). The tissue bicarbonate did not differ by more than 0.3 meq/kg H_2O .

Influence of the inspired oxygen concentration. This was varied in order to evaluate the possibility of tissue hypoxia during marked hyperventilation ($P_{\text{aCO}_2} < 25$ mm Hg). A comparison was made between the tissue CO_2 values measured in a group of 11 animals hyperventilated with air and those measured in another group of 29 animals hyperventilated with 50 per cent O_2 in nitrogen. In these and in following groups the individual values were corrected for difference in P_{aCO_2} by means of a parallel displacement of the points along a regression line calculated for the whole groups. The reference P_{aCO_2} value chosen was 15 mm Hg. The tissue CO_2 values in the two

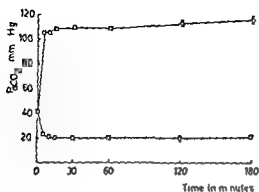


Fig 2

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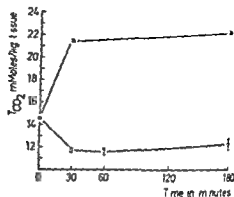


Fig 3

Fig 3 Changes in brain total CO_2 content during acute respiratory acidosis and baseosis. Rat pentobarbital or phenobarbital anesthesia. Exposure to CO_2 concentrations of about 15% (upper

groups were 11.2 ± 0.2 and 11.0 ± 0.1 meq/kg of tissue H_2O (mean \pm SE) respectively. The difference is not significant ($p > 0.1$).

The water content of the brain tissue did not show any significant variation with the anesthetic with the O_2 concentration of the inspired gas used or with the P_{aCO_2} level. The mean and SD from the 57 expts. used for deriving the CO_2 binding curve was 78.2 ± 0.6 per cent after correction for the difference in water content of the 3 per cent blood in the tissue.

Time course of changes in P_{aCO_2} and in the total tissue CO_2 content. An 'acute steady state' was defined from measurements of the P_{aCO_2} and the tissue CO_2 content at various times after a step change in the ventilatory state (see discussion). The step changes consisted of hyperventilation (300 ml per min, mean body weight 370 ± 10 g) or exposure to 15 per cent CO_2 . The P_{aCO_2} values were found to stabilize after 10–15 min. Fig 2 shows the response of two groups of 5 and 8 animals which were exposed to CO_2 or hyperventilated respectively. In hypercapnia a steady P_{aCO_2} level was recorded between 15 and 60 min. When the hypercapnia was maintained for 2 or 3 hrs there was an inconsistent further increase in P_{aCO_2} in three animals. In hyperventilation at constant volume the P_{aCO_2} level was constant from 10 to 180 min. The same general pattern was found in the intermediate P_{aCO_2} regions.

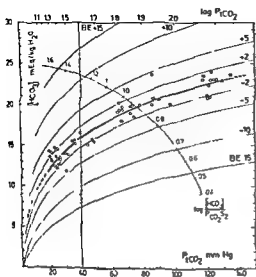


Fig 4

Fig 4 Relation between the mean bicarbonate concentration in brain tissue and the mean tissue P_{CO_2} (see text)

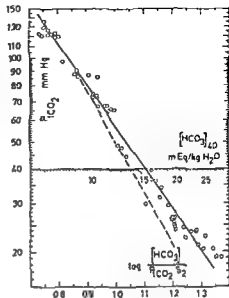


Fig 5

* variation of the buffer capacity (see text)

Fig 5 Relation between $\log P_{CO_2}$ and the logarithm of the buffer ratio ($\log [HCO_3^-]/P_{CO_2}$). The same material as in Fig 4. The solid line is the linear regression line of the present material. The broken line denotes the buffer capacity of cat brain tissue as calculated by Roos (1963) from data obtained with the DMO method.

The preliminary experiments showed that there were only small changes in the tissue CO_2 content from 30 to 180 min after a change in the ventilatory state (Ponten 1964). Experiments with the present technique verified the observation that the main change in T_{CO_2} occurred within 30 min. However, in hypercapnia, there was a slow further change in T_{CO_2} over the next 2.5 hrs, as shown in Fig 3. The additional change seen between 30 and 180 min was about 10 per cent of the entire change.

The CO_2 binding curve for brain tissue *in vivo*. On the basis of the above results an "acute steady state" was considered to be reached after 30 min. In order to establish the CO_2 buffering of the tissue over a wide P_{CO_2} range 57 expts were carried out at varying P_{CO_2} values, all rats being exposed to the respiratory change for 30 min. The results were plotted in a P_{CO_2} , HCO_3^- diagram (Fig 4). The regression line (thick line in Fig 4) was obtained by fitting a theoretically derived equation to the experimental data (see below). The regression line shows that the tissue bicarbonate concentration at a P_{CO_2} of 40 mm Hg was 16.0 meq/kg of tissue water, and further

that the tissue bicarbonate concentration increases from 12.5 to 22.5 meq/kg H_2O between the P_{CO_2} limits of 20 to 120 mm Hg. The 95 per cent confidence limits calculated for the whole material were ± 2.2 meq/kg H_2O .

Theoretical derivation of an equivalent CO_2 buffer curve Valuable information about the *in vivo* buffer curve can be obtained from a theoretical buffer system. Most conventionally, a one-compartment equivalent buffer system is considered which besides the CO_2 buffer system $-P_{CO_2} \cdot S_2 \cdot K_{H,CO_2} = (H^+) (HCO_3^-)$ — contains only one non-bicarbonate buffer system $-(HA) K_{HA} = (H^+) (A^-)$ — with a total buffer concentration of C mmoles/kg H_2O ($C = A^- + HA$). In a system where Na^+ stands for all cations except H^+ , and Cl^- for all nonbuffer anions, the following electrical neutrality equation will hold within 2 per cent for the pH range 5.0–7.8 (Siesjö and Pontén 1966 b)

$$(Na^+) - (Cl^-) = (HCO_3^-) + (A^-) = (BB) \quad (1)$$

In this equation (BB) is the buffer base concentration, i.e. the total buffer anion concentration (Singer and Hastings 1948).

After substitution from the equations given in the text above the following equation is obtained

$$P_{CO_2} = \frac{K_{HA} (HCO_3^-)}{K_{H,CO_2} S_2} \left(\frac{C}{(BB) - (HCO_3^-)} - 1 \right) \quad (2)$$

This equation contains 2 variables which can be obtained from the *in vivo* experiments, i.e. P_{CO_2} and (HCO_3^-) . Moreover, it contains the two constants $K_{H,CO_2} = 10^{-7.1}$ and $S_2 = 0.0314$ mmoles/kg H_2O /mm Hg and the three unknown parameters K_{HA} , C and BB . Derivation of a minimum buffer concentration (see below) requires that the most favourable K_{HA} is used. Since the midpoint of the experimental curve corresponds to a pH of 7.1 in a one-compartment system the most favourable K_{HA} should be $10^{-7.1}$ (maximal buffer capacity occurs when pK_{HA} equals pH, see van Slyke 1922). If this value of K_{HA} is inserted into the equation the remaining parameters C and (BB) would be defined from two points on the curve. Approximate values were obtained by pooling the observations into 5 groups. By inserting this approximate value for one of the parameters the other parameter could be calculated from each experiment. The regression equation obtained from these procedures was

$$P_{CO_2} = \frac{(HCO_3^-)}{0.314} \left(\frac{35}{36 - (HCO_3^-)} - 1 \right) \quad (3)$$

This equation, which not only fits the experimental points mathematically, but has a physicochemical meaning in that it describes the buffer capacity of a one-compartment buffer system, will make it possible to derive the following quantities:

1) *Minimum buffer concentration*, i.e. the buffer concentration needed to account for the buffer capacity of the tissue *in vivo* (Siesjö and Pontén 1966 b). At a K_{HA} of $10^{-7.1}$ the minimum buffer concentration is calculated to 35.1 ± 0.4 mmoles/kg H_2O . If the buffer acids which are responsible for the buffering of CO_2 have K_{HA} values differing from $10^{-7.1}$ an even higher buffer concentration is needed (see Discussion).

2) The buffer base concentration (BB) is calculated to 35.7 ± 0.2 meq/kg H₂O. If fixed acids or bases are added to the system they will cause a mole for mole change in BB. The amounts added will be the

3) Base excess values (BE = BB - 36) (Astrup *et al.* 1960) which express quantitatively the nonrespiratory acid base changes in the system. The base excess values have been entered into Fig. 4 as thin lines parallel with the buffer line, positive values expressing additions of base and negative ones additions of acid.

4) The standard bicarbonate value, i.e. bicarbonate concentration referred to a constant CO₂ tension (Astrup *et al.* 1960), is obtained for any experimental P_{iCO_2}/HCO_3^- pair by displacing the point along the buffer curve to the reference P_{iCO_2} (e.g. 40 mm Hg). The standard bicarbonate values are then read from the y axis. The standard bicarbonate change will express only part of the non respiratory acid base change, which is equal to BE. The ratio between the standard bicarbonate change and the base excess change is 0.7, i.e. an experimentally determined change in standard bicarbonate must be multiplied by 1.4 to give the amount of fixed acid or base which has been added to the system.

5) The buffer capacity of a system against CO₂ is most conveniently defined as

$$\beta_{CO_2} = d \log P_{iCO_2} / d \log (HCO_3^- / P_{iCO_2}, S_2) = d \log P_{iCO_2} / d \log BR \quad (4)$$

(Siesjö 1962 d), since the relation between $\log P_{iCO_2}$ and the logarithm of the buffer ratio ($\log BR$) is a nearly straight line. The buffer capacity can be obtained from the diagram of Fig. 4 in the following way. If two points along the buffer curve are joined with the origin by means of straight lines these lines will cut the $\log BR$ scale (circle sector in Fig. 4). $d \log BR$ is then given by the difference in $\log BR$ at the intersection of the $\log BR$ scale and the straight lines. $d \log P_{iCO_2}$ is the horizontal distance between the points on the buffer curve, read at the upper (logarithmic) P_{iCO_2} scale. The buffer capacity thus calculated from the experimental curve is 1.48 and equals the *in vitro* buffer capacity of whole blood containing 12 g hemoglobin/100 ml.

Although the buffer capacity can easily be calculated from the HCO_3^-/P_{iCO_2} diagram it is often visualized as the slope of a linear regression in the $\log P_{iCO_2}/\log BR$ diagram. Fig. 5 shows the present series in this plot. The solid line denotes the buffer slope. The regression equation was $\log BR = 2.183 - 0.674 \log P_{iCO_2}$. The broken line shows the buffer capacity obtained with an independent method. Roos (1965) (see Discussion).

Discussion

Experimental techniques. Accurate measurements of the total CO₂ content of the brain tissue will require a constant CO₂ tension and an adequate oxygen supply to the tissue during the fixation. About 60 sec are required to freeze the central parts of the brain in rats (Everett *et al.* 1956). The present freezing technique allowed at least the medullary respiratory and circulatory centers to function for about that time (45–60 sec) which supports the suggestions by Richter and Dawson (1918)

In no instance was the brain frozen *in situ*. Moreover, since the CO_2 concentrations of the gas mixtures used were unusually high, ranging from 24 % (Nichols 1958) up to 50 % (Brodie and Woodbury 1958) it is difficult to compare the results with the present data.

A systematic study of "intracellular pH" in brain tissue of the cat at physiological CO_2 tensions was recently published by Roos (1965), who calculated his values from distribution of the weak acid 5,5 dimethyl-oxazolidine-2,4-dione-2 Cl^- (DMO). The two methods, the CO_2 method and the DMO method, should principally give the same information, since both measure the distribution of weak acid in a system. The DMO method has the advantage that the tissue need not be frozen *in situ*, but it has the serious disadvantage that the concentration of the unionized form (HDMO) cannot be measured independently or adequately calculated. It is possible to calculate the mean capillary or the CSF HDMO concentrations, but because of the phenomenon of nonionic diffusion (Milne, Schriber and Crawford 1958) it is unknown whether the mean intracellular HDMO concentration corresponds to the calculated figures. Whereas the mean tissue CO_2 tension is well defined (Pontén and Siego 1966), the mean HDMO concentration is not, and the different assumptions used by Roos will give large variations in the bicarbonate concentrations calculated. The DMO data of Roos show a buffer capacity of at least 1.8, i.e. much higher than that calculated from the present CO_2 data. This is principally the same difference between the two methods as was obtained by Miller, Tyson and Relman (1963) applying both methods to isolated rat diaphragm (see Siego and Pontén 1966 b).

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Catecholamine Excretion in Adreno-Demedullated Rats Exposed to Cold after Chronic Guanethidine Treatment

By

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Abstract

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Adreno-demedullated rats subjected to a moderate cold exposure showed a significant increase in urinary excretion of noradrenaline (NA) and maintained their thermal equilibrium. Adreno-

Exposure to cold is known to produce an important and almost immediate increase in urinary excretion of noradrenaline (NA) (Leblanc and Nadeau 1961, Leduc 1961). Other studies have shown a markedly diminished resistance to cold of rats whose catecholamine stores have been depleted with reserpine (Dandija, Johnson and Sellers 1960, Zilberstein 1960, Taylor 1961). A closed relationship between cold resistance and urinary excretion of NA was obtained in such treated animals (Johnson 1963). These results and others indicate an important role of NA in the survival of rats subjected to cold. However, it is difficult from these studies to determine if the decreased resistance is due mainly to a peripheral or central action of reserpine, since this drug has important depleting effects on both brain catechol and indolamines (Sheppard and Zimmerman 1960) and besides produces a marked sedation.

In order to evaluate the importance of the peripheral liberation of catecholamines, we have used guanethidine, a potent sympatholytic drug, which blocks nervous transmission in the noradrenergic postganglionic fibres (Maxwell *et al.* 1960) and depletes various peripheral organs of their catecholamine content without affecting amine stores in the brain (Kuntzman *et al.* 1962). It is a strongly basic compound

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TABLE 1 Noradrenaline content of organs after chronic injections of guanethidine (10 mg/kg i. p.) daily for one week. Numbers in parentheses indicate the number of animals in each group

	Control (3)	Guanethidine (4)
	$\mu\text{g/kg b. w.}, \text{mean} \pm \text{S.E.}$	$\mu\text{g/kg b. w.}, \text{mean} \pm \text{S.E.}$
Heart	0.78 ± 0.030	0.11 ± 0.043
Spleen	0.83 ± 0.032	0.092 ± 0.024
Brain	0.40 ± 0.030	0.39 ± 0.013

having such a low lipid solubility that it does not cross the blood brain barrier to an appreciable extent (Kuntzman *et al.* 1962). In addition, guanethidine, contrary to reserpine, does not impair the resistance of rats exposed to very low temperatures (Pouliot and Leblanc 1963). In the present work it is attempted to evaluate more precisely the importance of peripheral secretion and action of NA in the defense against cold.

Materials and Methods

All experiments were carried out in the laboratory of the University of Montreal, Canada. The animals were placed in individual metabolism cages coated with plastic to prevent oxidation by metallic ions. The cages were mounted on polyethylene funnels at the bottom of which were placed small round bottom flasks placed upside down over a 100 ml beaker to discard the feces. Acetic acid 1 N was added to the urine so that the pH was maintained around 3. Urine collection was started in the morning and continued for the next 24 hrs. Each urine specimen was filtered and assayed for free adrenaline (A) and (NA) according to the method of Euler and Lishajko (1961).

In order to determine catecholamines in organs rats were killed by a blow on the head and the organs immediately removed, rinsed with cold saline, weighed and extracted with trichloroacetic acid (10%). The organs were then assayed for A and NA in essentially the same manner as the urine samples. A Turner fluorimeter model 110 was used for the determinations.

Body temperatures were measured by inserting a thermocouple 4–5 cm inside the rectum. Guanethidine (Ismelin[®] CIBA) was prepared in an aqueous solution of 10 mg/ml and injected intraperitoneally in a dose of 10 mg/kg/day (expressed as the free base).

Results

Preliminary experiments showed that there is no change in brain catecholamines of rats injected with guanethidine daily for one week whereas there is a 85 to 90% depletion in heart and spleen (Table 1).

Adrenal demedullation was followed by a moderate fall in body weight (15–20 g) of the rats. Within a week they were back to their initial weight and guanethidine treatment did not cause any further loss in body weight throughout the entire period of treatment. The treated rats were not sedated, but showed a mild and transient diarrhea.

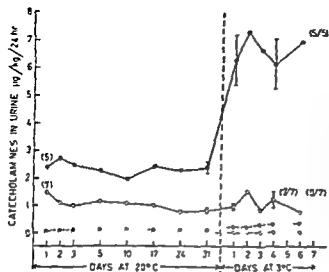


Fig 1 Urinary excretion of catecholamines from adreno-demodulated rats exposed to 20°C and 3°C. Numbers in parentheses indicate the number of animals in each group at 20°C and number of rats surviving at 3°C. Standard errors are plotted on day 31 at 20°C and days 1 and 4 at 3°C.

— — — Noradrenaline
 — — — adrenaline ● Control rats
 ○ Guanethidine treated rats

The urinary excretion of A and NA of control and guanethidine treated animals placed first at 20°C and then at 3°C is shown in Fig 1. Urinary NA showed a 50% decrease in the treated group kept at 20°C. $P < 0.01$. Adrenaline excretion which was low in the control adreno-demodulated rats could not be determined in the treated group. Upon exposure to 3°C, the control rats significantly increased their NA excretion $P < 0.05$ within the first 24 hrs and maintained this level for the duration of the experiment. Adrenaline excretion was also slightly increased in these animals. On the other hand the guanethidine treated group failed to show a significant increase in the urinary excretion of A and NA. Six days later the injected rats became hypothermic and died within 48 hrs. At this time the control adreno-demodulated rats were still all normal and normothermic.

Discussion

The general level of activity is one of the factors regulating body temperature and most drugs with a depressing action on the central nervous system at the same time affect the regulation of the body temperature (Euler 1964). It is therefore quite important that a drug should be free of central effects if the correlation between NA secretion from the peripheral tissue stores of catecholamines and resistance to cold stress is to be considered. According to Kuntzman *et al.* (1962), the action of guanethidine is largely peripheral and does not affect the brain NA content. Some authors reported depletion in brain NA after guanethidine (Kronenberg and Schumann 1962; Pfeiffer, Vizi and Satoru 1962) and it is difficult to explain this discrepancy except that there could be strain differences. However even in these reports the depletion is quite moderate and no overt behavioural changes are observed. In our experiment guanethidine treatment for 7 days had no effect on the NA content in the central nervous system (Table I).

As shown in Fig. 1, the urinary output of NA is significantly decreased in chronically guanethidine treated adreno-demodulated rats kept at 20° C. Moreover, if the same group is exposed subsequently to 3° C, no significant increase in NA excretion was noted and all died 7 days later, only a few hours apart. The control animals, in contrast, upon exposure to 3° C showed a threefold increase in urinary NA within the first 24 hrs, which was maintained throughout the duration of the experiment. No death occurred in this group and all animals were still normothermic at the end of the experiment. Since no change in the NA tissue content was noted in the control, the increase in the NA excretion following cold stress is likely to be the result of an increased release, followed by resynthesis. The failure of the guanethidine treated rats to increase their NA excretion suggests that guanethidine prevents either the increase in release or the biosynthesis or both, normally induced by cold exposure.

Acceleration of catecholamine synthesis following an increased nervous stimulation has been reported in the adrenal medulla (Hököfelt and McLean 1950, Bygdemann, Euler and Hököfelt 1960). It is likely that the same mechanism occurs in noradrenergic fibres. The sustained increase in urinary excretion of NA in cold exposed rats in the presence of an unchanged NA content in tissues (Leduc 1961), strongly suggests increased synthesis of NA as a result of increased nervous activity. On the other hand, it is well known that guanethidine blocks the response of postganglionic noradrenergic fibres to nerve stimulation (Maxwell *et al* 1960, Cass and Spriggs 1961, Boura and Green 1962) apparently by a persistent depolarization of the noradrenergic presynaptic terminals (Chang, Costa and Brodie 1965). Our results would then indicate that the sympathetic blockade can be partly responsible for the lack of acceleration of catecholamine liberation. The present results also corroborate those of previous investigators in which it has been shown that cold resistance is dependent upon the liberation and action of NA (Hsieh, Carlson and Gray 1957, Leduc 1961, Johnson 1963).

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The Effect of Various Types of Fat on the Cholesterol Distribution in the Rat

By

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Abstract

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and above the effect of oleic acid. The carcass cholesterol of rats given corn oil was slightly lower than in rats given type 2 of fat. The difference was statistically significant on cholesterol free diets. When various tissues were analysed corn oil induced transfer to other tissues than the liver could not be observed.

The fact that polyunsaturated fats reduce the plasma cholesterol level in man and animals is well established, likewise that it is a lasting effect. The cause of this cholesterol lowering effect is under vigorous discussion, and a number of observations seemingly at variance with each other have been reported. In many instances however, with what present day workers (Eneroth, Hellstrom and Ryhage 1964; Miettinen, Ahrens and Grundy 1965) consider to be inadequate methods extra excretion of bile acids and sterols in feces have been reported following administration of corn oil to hypercholesterolemic man or animal. Miettinen *et al.* (1965) have extensively reviewed previous work.

Most recent work in the field (Spritz, Ahrens and Grundy 1965; Avigan and Steinberg 1965; Bieberdorf and Wilson 1965) have failed to confirm these earlier reports.

It has been suggested by many that cholesterol is transferred from plasma to the tissues following ingestion of corn oil. When corn oil has been compared with saturated

fat a reduced plasma cholesterol level with a simultaneous considerable increase in liver cholesterol in the corn oil fed animals has frequently been observed (in rats by Grunbaum *et al* 1957, Avigan and Steinberg 1958, Reiser *et al* 1963, but not by Okey and Lyman 1957, in cebus monkeys by Wissler *et al* 1963) Moore and Williams (1964), on the other hand, found distinctly lower plasma and liver cholesterol in rabbits given a corn oil diet than in rabbits given a saturated fat diet. The level of fat in the diet in the above experiments was 15–30 per cent and the experiments lasted from 2 weeks to many months. In man Iranz and Carey (1961) found that plasma and liver cholesterol was reduced by 9 and 25 per cent, respectively, when 5 healthy men with high normal levels of plasma cholesterol received 3 ozs of corn oil daily for 1 month. In a parallel experiment in men given hydrogenated coconut oil the result was a small but insignificant increase in plasma and liver cholesterol.

It thus seems to be well established that polyunsaturated fatty acids can induce redistribution of cholesterol from plasma to liver, but it does not seem to be a constant occurrence.

In one series of experiments Gerson, Shorland and Adams (1961) have analysed a number of tissues from adult rats given 11% corn oil in the diet and compared the results with data found in rats given a diet poor in fat. They concluded that corn oil transferred cholesterol to various tissues even to the arterial wall.

Since their conclusions has attracted wide attention we felt that a repetition and extension of such types of experiments in rats was needed not least in view of the fact that Hauge and Nicolaysen (1959) had found that plasma and liver cholesterol in hypercholesterolemic rats was rapidly reduced by 80 mg soybean oil daily. A number of similar experiments has been performed by us confirming and extending the 1959 results (Gran and Nicolaysen 1966).

Experimental

Two series of experiments were conducted.

Approximately one year old hooded and albino female rats were taken from the interbred strains of this institute. The rats had previously been fed a stock diet containing 7.5% arachis oil in addition to the basal diet. The fat content of this

was substituted for fat and cholesterol. The rats were then divided into groups and given diets with different combinations of hydrogenated coconut fat, corn oil and olive oil as appears from the tables. The diet of Hauge and Nicolaysen (1959) was again used but the fat content was increased to 20 per cent at the expense of the equal weight of sucrose. One per cent cholesterol was added to the diets when desired.

The rats were given their respective diets for the six weeks following. The rats were next stunned

Analytical Methods

All chemicals were of analytical quality with the exception of the solvents which were of laboratory reagent quality and they were always redistilled before use.

TABLE I Comparison of cholesterol methods Liver cholesterol, mg/100 g

Group	Per cent fat in diet	Number of rats	Method		
			Tschugaeff ¹	Liebermann Burchard ²	Sperry-Webb ³
A Coconut fat ⁴	19.5	8	550 ± 56	322 ± 20	293 ± 23
Corn oil	0.5				
B Coconut fat ⁴	19.5				
Corn oil	0.5	9	3,340 ± 449	3,369 ± 524	3,311 ± 492
Cholesterol	1.0				
C Corn oil	20.0	10	494 ± 39	293 ± 14	252 ± 12
D Corn oil	20.0				
Cholesterol	1.0	8	5,759 ± 255	5,731 ± 255	5,475 ± 227

The values are means ± standard error of the mean

¹ Hauge and Nicolayssen (1938)

² Cook (1938)

³ Sperry and Webb (1930)

⁴ Fully hydrogenated

Tissues were weighed out in duplicate for extraction of the lipids

The lipids were extracted with mixtures of chloroform-methanol in the proportion 1:2 in the first extraction followed by two extractions where the solvent proportions were 1:1 and 2:1 respectively. A solvent volume in ml corresponding to 15–20 times the tissue weight in g was used in each extraction. The extracts were heated to boiling on a water bath and thereafter left at room temperature with occasional swirling for the next 30 min. The residue was filtered off, washed with fresh solvent on the filter, and resuspended in the next solvent mixture.

The combined extracts were washed with saline in a separating funnel using a volume of about one fifth of the total solvent volume. The solvents were then removed in a rotary evaporator and the residue was taken up in chloroform and diluted if necessary to the desired volume after filtration.

The skin was very difficult to homogenize and was therefore left over night in 2% KOH at room temperature. Complete dissolution resulted and the sterols could readily be extracted with ether.

Recovery studies with added cholesterol indicated that no loss occurred in such a procedure.

Cholesterol analyses. Serum cholesterol was analysed with the Hauge-Nicolayssen method (1938) which uses the Tschugaeff reagents following saponification. In work to be published later we have in the laboratory found that the Hauge-Nicolayssen and the Sperry-Webb procedure yield results which do not differ significantly in serum analyses. However, when the Tschugaeff principle was applied in analyses of various tissues we found that other substances interfered. A systematic study of cholesterol analysis of tissues followed. The methods used were: 1. Liebermann-Burchard reaction directly after saponification as described by Cook (1938); 2. the same reaction following precipitation with dithionite (Sapountzis et al., 1953); 3. the same reaction following

TABLE II Comparison of cholesterol methods. Carcass cholesterol, mg/100 g

Group	Per cent fat in diet	Number of rats	Method			
			1	2	3	4
A Coconut fat ¹	19.5	5	247±21	186±3.0	167±1.5	161±6.4
Corn oil	0.5					
B Coconut fat ¹	19.5	5	261±17	223±5.9	184±2.1	188±2.9
Corn oil	0.5					
Cholesterol	1.0					
C Corn oil	20.0	5	285±15	193±9.4	168±3.4	169±7.0
D Corn oil	20.0	5	312±17	225±10.2	183±5.4	189±9.8
Cholesterol	1.0					

The values are means ± standard error of the mean

Method 1 Tschugaeff reaction Hauge and Nicolaysen (1958)

2 Liebermann Burchard reaction Cook (1958)

3 Liebermann Burchard reaction after digitonin precipitation Sperry and Webb (1950)

4 Anthrone reaction with digitonin Goodman et al (1963) and Gna (1955)

¹ Fully hydrogenated

Results

Data showing a comparison of methods of cholesterol analysis in various tissues are presented in Table I and II

Liver Only the LBR digitonin method seems to give the true value of cholesterol in livers with normal cholesterol content. The direct Liebermann Burchard reaction gave results 10–20 per cent higher, and the Tschugaeff method results which indicate that the reagent used reacts with other material to give erroneously high values. However, when the liver cholesterol has increased to several per cent the Tschugaeff method gives an approximately correct value for liver cholesterol.

Carcass The difference between the direct LBR and the digitonin LBR was approximately that found with liver. With regard to the Tschugaeff reaction the values are again too high. The two methods of analysis of the digitonin precipitate showed full agreement between analysis of cholesterol and of the digitonide with anthrone. The results exclude the presence of saturated 3-β hydroxysterols which produce no colour in the LBR.

For the results recorded in subsequent tables, serum was analysed using the

A and C should first be compared since they represent corn oil versus saturated fat. For all samples analysed the values are lower in the corn oil group, however, signifi-

TABLE III Cholesterol in adult rats Skin and carcass mg/100 g

Group	Per cent fat in diet	Number of rats	Skin Sperry Webb	Carcass	
				Liebermann Burchard	Sperry Webb
A Coconut fat ¹	19.5	12	226 ± 7.3	190 ± 3.4	172 ± 3.0
Corn oil	0.5				
B Coconut fat ¹	19.5	11	225 ± 5.4	212 ± 4.3	180 ± 3.4
Corn oil	0.5				
Cholesterol	1.0				
C Corn oil	20.0	12	226 ± 9.4	190 ± 4.3	163 ± 3.2
D Corn oil	20.0	12	226 ± 11.8	217 ± 7.2	177 ± 5.7
Cholesterol	1.0				

The values are means ± standard error of the mean

¹ Fully hydrogenated

² Group A versus group C $p < 0.05$

TABLE IV Cholesterol in adult rats Serum and liver

Group	Per cent fat in diet	Number of rats	Serum mg/100 ml Tachugaeff	Liver mg/100 g	
				Liebermann Burchard	Sperry Webb
A Coconut fat ¹	19.5	12	74 ± 5	319 ± 23	285 ± 24
Corn oil	0.5				
B Coconut fat ¹	19.5	11	195 ± 18	3369 ± 524	3311 ± 492
Corn oil	0.5				
Cholesterol	1.0				
C Corn oil	20.0	12	67 ± 4	299 ± 14	261 ± 12
D Corn oil	20.0	12	292 ± 6	6280 ± 349	5922 ± 360
Cholesterol	1.0				

The values are means ± standard error of the mean

¹ Fully hydrogenated

TABLE V. Cholesterol in adult rats

Group	Per cent fat in diet	Number of rats	Body weight g	Serum cholesterol ¹ mg/100 ml
1 Coconut fat	19.5	12	245.3 ± 6.9	161 ± 19.5
Corn oil	0.5			
Cholesterol	1.0			
2 Corn oil	20.0	12	213.8 ± 6.7	262 ± 18.9 ²
Cholesterol	1.0			
3 Coconut fat	12.0	12	236.3 ± 6.4	336 ± 22.7 ³
Olive oil ⁴	0.0			
Cholesterol	1.0			

¹ Tschugaeff reaction Hauge and Nicolaysen (1958)² Sperry and Webb (1950)³ Skin with hair

cantly so only for carcass cholesterol. After addition of cholesterol to the two types of diets considerable hypercholesterolemia developed and the livers contained several per cent of cholesterol. A slight increase was observed in carcass cholesterol representing 4.7 per cent in the saturated fat group and 8 per cent in the corn oil group. However, the values for the corn oil group do not exceed those for the coconut fat group.

The data for the skin free of hair are surprisingly identical for the 4 groups, indicating that cholesterol is not readily accumulated in the skin.

The amount of cholesterol accumulated in the liver constitutes several hundred mg, whereas the group difference for the carcasses are in the range of 10–25 mg per rat weighing 250 g.

The increased liver serum cholesterol ratio in group D compared with group B (21.7 versus 17.3) corresponds to results reported by earlier workers. The high level of cholesterol in group D we suspected was due to the well known effect of unsaturated fatty acids on cholesterol absorption (Bloomfield 1964, Leveille and Sauberlich 1964).

A second series of experiments was designed to bear on this point. As mentioned above, the oils added to the diets in groups 2 and 3, Table V, were added in amounts calculated to ensure that the oleic acid concentrations of the diets were identical. In the preceding series all the material representing various tissues had been exhausted in the work on the comparison of cholesterol methods. We therefore included in this series also analyses of various tissues. Individual analysis was performed with liver and serum only, elsewhere the samples were pooled and analysed as one single sample representing all animals in each group.

Cholesterol^a, mg/100 g

Liver	Aorta	Pelt ^a	Muscle	Intestine	Carcass ^a
1 760 ± 170	161	310	70	276	204
6 970 ± 510 ^b	157	320	71	302	196
4 900 ± 350 ^b	198	310	73	303	210

^a Carcass minus liver, aorta, skin intestine and muscle sample^b Group 2 versus group 3 Serum cholesterol $p < 0.001$, liver cholesterol $p < 0.001$ ^c The oleic acid content was equal in the diets of group 2 and group 3

The results obtained with regard to serum and liver cholesterol in groups 1 and 2 are in line with those of the first series. The expected effect of oleic acid seems to be obtained. The average cholesterol content of the livers in groups 2 and 3 was 746 and 473 mg respectively. The results indicate that cholesterol absorption is enhanced by linoleic acid over and above the effect of oleic acid. The high liver plasma cholesterol ratio in group 2 compared with group 3 again emphasizes the role of linoleic acid in the redistribution of cholesterol from plasma to the liver.

The minor differences observed between other results shown do not appear to warrant too much emphasis. Again the carcass cholesterol is lowest in the corn oil group, the data for aorta being inconsistent when seen in relation to plasma cholesterol concentration. The higher values for the intestine in the groups given oils may reflect an increased concentration due to increased rate of absorption.

The cholesterol values for the skin in this series are distinctly higher in the first series, and this should be so since the skin was analysed with hair in these animals. Five samples of hair from normal animals were analysed and the cholesterol content varied from 0.7 to 1.2 per cent. We have not studied the problem of cholesterol transfer through the skin to the hair, and we do not therefore know whether the higher cholesterol values of the latter may in part represent transfer of this type. However, there is no indication of increased cholesterol concentration due to a corn oil effect.

Discussion

The results with regard to cholesterol distribution between plasma and liver in the rats given dietary cholesterol are in line with the results of many previous studies.

in which an increased liver/plasma cholesterol ratio was caused by corn oil. On the other hand, we could find no indication of a redistribution to other tissues, on the contrary, the carcass cholesterol was significantly lower in the group given 20 per cent corn oil in the diet than in the group given 19.5 per cent coconut fat and 0.5 per cent corn oil. Bloomfield (1964) compared the effects of butter and safflower oil (— 20 per cent fat in the diet —) on cholesterol distribution in rats given 0.65 per cent of cholesterol in the diet. He found no indication of an increase in cholesterol concentration in other tissues than the liver, results in line with ours. When carcass including liver was analysed in rats given cholesterol in the diet, obviously greatly increased values for carcass cholesterol are found (Wilson 1962, Tidwell, McPherson and Burr 1962). On the other hand our results are at variance with those reported by Gerson *et al.* (1961). We compared rats given two types of fat in the diet while they compared data from rats given corn oil with those from rats given a diet very poor in fats. They report data for free and esterified cholesterol, and the results with regard to 'transfer' of the two cholesterol fractions are inconsistent *e.g.* in aorta only free cholesterol increased in the pelt only ester cholesterol. No data for variability are given in their paper. The increase in aorta cholesterol — 39 mg per 100 g tissue — corresponds to 0.034 mg per 80 mg. We find the approximate weight of a rat aorta to be 60–80 mg. In our analyses of pooled aorta from 12 rats the values for the corn oil group came out as the smallest (Table V) and do not thus support the view of transfer of cholesterol from plasma to aorta mediated by corn oil. It has been amply demonstrated that the deposition of cholesterol in the arterial wall occurs roughly in proportion to plasma cholesterol in experimental animals. Until the same type of results as those reported by Gerson *et al.* has been produced in new and extended experiments it seems justified to doubt the biological significance of the type of transfer postulated by these authors. Recently, Bieberdorf and Wilson (1965) induced hypercholesterolemia in rabbits by feeding them on a diet containing hydrogenated coconut oil. When thereafter corn oil was substituted for the saturated fat, plasma cholesterol was reduced as expected, but no effect on synthesis or degradation of cholesterol was found. Samples of muscle were collected from the 10 rabbits used, and a nearly constant but statistically not significant increase in muscle cholesterol was found. The liver was not analysed; however, the contention of these workers being that cholesterol is transferred from plasma to a great muscle mass in a period of declining plasma cholesterol concentration. Such a transfer would imply a change of the membrane of muscle cells so as to increase its content of cholesterol or it would imply an active transport. Clearly much better evidence must be produced before such an hypothesis can be accepted. As discussed by us (Gran and Nicolaysen 1966) extra sterol excretion induced by corn oil has been found in some experiments, but not in others. It then seems reasonable to assume that under such circumstances a redistribution to the liver temporary or permanent has taken place.

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Cod Liver Oil Induced Removal of Cholesterol in Hypercholesterolemic Essential Fatty Acid Deficient Rats

By

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Abstract

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Rats were given a diet free of polyenoic fatty acids and containing 1 per cent of cholesterol until 150 days old. Severe hypercholesterolemia and livers containing several per cent of cholesterol resulted. Next 80 mg of cod liver oil was given daily to each rat for 14 days. The result was that about 28 mg of cholesterol disappeared from a rat body per day. The pathways responsible were not further studied.

The effects of various types of fats, included in substantial proportions in the diet, on the distribution and metabolism of cholesterol have been discussed elsewhere (Gran and Nicolaysen 1966). When the level of fat in the diets is 10—40 per cent, numerous mechanisms known to be active in cholesterol metabolism can come into play. We refer to a recent discussion by Avigan and Steinberg (1965) and to Gran and Nicolaysen (1966).

In the present work we have used another type of experimental design. This followed the pattern common in bioassays, namely the use of animals free of the substance to be tested. The dose response curve in such assays is logarithmic. The general experience is that the most reliable results are obtained when small doses of the substance to be tested, mostly vitamins, result in a high degree of response. In bioassays of vitamin D in rats, we find that doubling the dose from 0.4 to 0.8 units nearly doubles the intensity of cure, whereas an increase to 1—2 units results in only a small extra response. Hauge and Nicolaysen (1959) designed a method of this type to study the hypocholesterolemic effects of various types of polyunsaturated fatty acids. The present work is a repetition and an extension of the early experiments. Since the effect of the polyenoic acids of cod liver oil in depressing serum

TABLE I The effect of 80 mg cod liver oil daily on serum and liver cholesterol in hypercholesterolemic rats free of essential fatty acids

Group	I	II	III		IV		
	Control	Cod liver oil	Control		Control		
Number of rats	3	5	6		5		
Sex	2 m, 3 f	4 m, 1 f	5 m, 1 f		5 f		
		0 day	14 days	0 day	14 days	0 day	14 days
Age, days	146	146	160	212	226	86	100
Body weight, g	128± 8.6 ¹	124± 7.5	163± 4.8	133± 4.9	132± 4.4	103± 4.1	107± 6.6
Serum cholesterol mg/100 ml	541± 24	518± 30	356± 34	543± 48	555± 77	386± 18	355± 48
Liver cholesterol mg/100 mg	9,000± 469	8,150± 630 ¹	4,530± 598	4,820± 750 ¹	4,720± 444	4,090± 598 ¹	5,131± 108
Liverfree carcass mg/100 g	321± 21		263± 10				

All analyses by the Tschugaeff reaction (Hauge and Nicolayzen 1958)

¹ The values are means±standard error of the mean

² Liver biopsy

cholesterol was found to be about 4 times that of linoleic acid in vegetable oil (Nicolayzen and Ragaard 1961) cod liver oil was used as the source of polyenoic acids

Experimental

Flooded and albino rats from the same stock as used in the

rats continued on the same diet after weaning and until the experiments were concluded the rats were killed at the ages given in the table

A small blood sample was taken from the tail for the determination of serum cholesterol and it was to be used when necessary for the determination of liver cholesterol

the administration of cod liver oil was started The left half of the median liver lobe was removed while the rats were kept in ether anaesthesia the amount of tissue removed corresponded to 1 g of liver) Since the cholesterol concentration was taken

the blow over the neck or the methods for tissue analyses have been described by Grant and Nicolayzen (1956)

Results

The results appear from Table I. Serum and liver cholesterol were rapidly reduced in the course of the 2 weeks experiment. In the control groups (III and IV) the average values for serum and liver cholesterol remained largely unchanged, indicating that the removal of about 1 g of liver does not affect the cholesterol concentration of the liver in the course of 14 days.

The difference in cholesterol concentration in plasma and liver between the 3 groups need some comments. The animals used in experiments of this type are selected from a much larger group of rats in which serum cholesterol is analysed the day before the actual experiment is started. The selection aims at groups with the same average serum cholesterol level. In group II the serum cholesterol level was initially the same as in group III, the liver cholesterol, however, was nearly twice that in the experimental group. We have in the course of years found that variation within litters and between litters is considerable, and it appears from Table I that serum and liver cholesterol do not rise in parallel. In the earlier experiments (Hauge and Nicolaysen 1959) serum cholesterol was 425 mg/100 ml and liver cholesterol 3.4 per cent on zero day. Following two weeks' dosing of 80 mg soybean oil daily, the values were reduced to 191 mg/100 ml and 1.92 per cent respectively.

Thus, group averages vary from time to time in rats given these hypercholesterolemic diets. However, this does not weaken the conclusion that cod liver oil induced a reduction of serum and liver cholesterol.

The cholesterol concentrations of various tissues and in the carcass were determined, only the values for carcass cholesterol being reported. The reason is the following. The experiments presented here were done before the experiments in adult rats (Gran and Nicolaysen 1966) were started. The erroneously high tissue cholesterol values obtained with the Tschugaeff method have been documented by us. Somewhat lower values were found for the cholesterol concentration in tissues analysed in the cod liver oil group than were found in the control group. The livers weighed on an average 11 g in the cod liver oil group, and thus contained about 0.9 g of cholesterol of which 0.4 g was removed in the course of 14 days. The rats minus liver weighed about 150 g at the end of the experiment. A transfer of the cholesterol from the liver to the carcass would imply an increase of cholesterol from 265 to 530 mg per 100 g carcass. In consequence we feel justified in concluding that in the course of the experimental period cholesterol removed from the liver was also removed from the rat. Our negative findings (Gran and Nicolaysen 1966) with regard to transfer of cholesterol to other tissues than the liver lend support to this conclusion.

Discussion

The cod liver oil used contained about 25 mg of polyenoic fatty acids chiefly of the penta- and hexaenoic type. Under the present experimental conditions a mean disappearance of about 28 mg of cholesterol daily from a rat body was observed. The effect of fats rich in polyunsaturated fatty acids on the distribution of cholesterol

in rats and other species when the diets contain 10—30 per cent of such fats has been discussed elsewhere (Gran and Nicolaysen 1966). In the recent years a number of studies on the metabolism of cholesterol under the influence of diets relatively rich in polyunsaturated fatty acids have been published and a brief discussion of the results is needed.

In their work on rats Avigan and Steinberg (1958) found evidence of increased synthesis of cholesterol in the liver under the influence of corn oil. On the assumption that steady state prevailed in their rats, the data also indicated increased excretion or breakdown of cholesterol. Lewis, Pilkington and Hodd (1961) injected plasma lipoproteins containing ^{14}C labelled cholesterol from donor rats into other rats. Bile acids and sterol excretion by the recipient rats increased when an unsaturated fat was given to the donor rats.

Eneroth, Hellestrom and Ryhage (1964) studied the excretion of fecal sterols in 7 men fed a standardized diet supplemented with butter fat or corn oil. Only in 3 of the 7 men were feces collected continuously when they were switched from the butter (14—18 days collection) to the corn oil period (23—32 days collection). The extra excretion of cholesterol and cholesterol neutral metabolites per day in the corn oil period was 63, 122 and 172 mg per day, respectively, in 3 men. The cholesterol intake in the butter period was 250 mg, in the corn oil period 130 mg daily. According to Spritz, Grundy and Ahrens (1963) true absorption of cholesterol in man is 62—86 per cent on a cholesterol free diet. When dilution of endogenous cholesterol with exogenous cholesterol occurs, true absorption will be reduced and, assuming that 70 per cent of the 120 mg cholesterol ingested in excess in the butter period was absorbed, 36 mg must be added to the above figures for extra excretion of cholesterol in the corn oil period. It must be concluded therefore that corn oil caused a highly significant extra excretion of cholesterol in the 3 men. In the oil period collection did not start until 14—16 days after the beginning of the corn oil period and the collection periods were correspondingly shorter and more irregular results were obtained.

In the recent studies by Spritz, Ahrens and Grundy (1965) and by Avigan and Steinberg (1965) in a total of 12 men a significant extra excretion of sterols was obtained in 2 only.

The variability and uncertainties inherent in balance studies are well known and Sjogren (1964, 1965) have recently made important contributions to the discussion of this subject. In this laboratory we have over decades gained considerable experience from our studies of calcium metabolism in man and animal. A brief discussion may be useful. When feces are collected over 2 weeks the sampling error is not zero. Markers are needed to reduce the error. A marker, e.g. TiO_2 , should be used in one period and another, in the other when results from 2—3 weeks' experiments are compared. In long term calcium balance studies by Malm (1958) in man the feces collected over 2 weeks could vary several hundred mg from one period to the next, the calcium content of the diet being constant.

variations occurred lasting for several 2 week periods in which positive balances alternated with negative ones due to the variability in fecal calcium. Similar results have been reported for calcium by Ackerman and Toro (1953) and for magnesium by Seelig (1964). Over 40–50 days were reported negative balances of such a magnitude that, were they representative of the calcium and magnesium balance of the persons studied, all calcium and magnesium would have been lost from their bodies in the course of 1–2 years. The reason for such results is not clear. In studies of digestive juice calcium in rats Gran (1960) found a variability of about 100 per cent occurring in rats from week to week when the food intake was constant.

Although much remains to be elucidated, the fact is that significant extra sterol excretion under the influence of corn oil has been found in 5 out of 15 men. A temporary redistribution of cholesterol from plasma to the liver can explain negative findings. Provided synthesis, degradation and excretion do not change when a subject is transferred from a saturated to an unsaturated fat diet such a redistribution may conceivably become permanent. It remains a matter of opinion whether the present finding should be taken as evidence of a catabolic (excretion and/or degradation) effect of polyunsaturated fatty acids in cholesterol metabolism in general, irrespective of the amount of fatty acids given. Conceivably only small amounts of polyenoic fatty acids may under certain circumstances be required for a normal (maximal?) sterol excretion and/or degradation. When oils are given in quantities of 10–30 per cent in the diet the interplay between effects of unsaturated fatty acids on absorption and reabsorption, on synthesis, degradation and redistribution between plasma and liver may decide the net result in a given experiment.

More detailed studies in man and animals are needed to establish the true picture of sterol balances when the type of dietary fat is altered. We concur with the view of Avigan and Steinberg (1965) that there is at least no necessary and consistent relationship between fat induced changes in serum cholesterol levels and rates of fecal sterol and bile acid excretion. A conclusion to the effect that corn oil does not increase fecal sterol excretion, and therefore transfer cholesterol to the tissues is not justified in view of present day pooled observations.

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Studies on the Ganglionic Site of Action of Sympathetic Outflow to the Stomach

By

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Abstract

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In acute experiments on cats anesthetized with chloralose inhibition of excitatory vagal responses of gastric smooth muscle was produced by reflex or direct activation of an adrenergic nerve fibre system. The reflex that could be elicited by distension of a jejunal loop or stimulation of mesenteric afferents was dependent on intact spinal connections because it was blocked by spinal anesthesia. Activation of the adrenergic nerve fibre system did not inhibit gastric myogenic tone or gastric muscle contraction elicited by the muscarinic action of acetylcholine. It was therefore concluded that the adrenergic outflow responsible for this type of inhibition acts on parasympathetic intramural ganglia or cells involved in the excitatory control of gastric motility.

According to Bayliss and Starling (1899), the intestines are normally under the reciprocal control of vagal excitatory and splanchnic inhibitory fibres. This postulated arrangement has been generally assumed to hold also for the stomach though no recent studies on this organ seem to have been published (for details regarding earlier work see McSwiney 1931). But observations by Celander (1959) and Kock (1959) on intestinal motility suggest that the inhibitory influence of a supraspinal reflex increase in sympathetic activity is not caused by specific inhibitory fibres but by a reflex release of catecholamines from the adrenal medulla. These observations were difficult to bring into line with the view that specific inhibitory fibres act directly upon the intestinal smooth muscle cells.

The so-called intestino-intestinal inhibitory reflex (Kuntz and Saccomanno 1944, Kock 1959), on the other hand, fulfils most criteria of a direct nervous mechanism, in that it is prompt in onset and can easily be elicited even when the adrenal glands have been removed. Johansson and Langston (1964) showed that it is a true propriospinal reflex, easily evoked in the acute spinal preparation and normally exposed to a more or less dominant tonic inhibitory influence of supraspinal origin (Johansson, Jonsson and Ljung 1965). This arrangement differs from the sympathetic cardiovascular control, where the spinal "centres" are normally facilitated by a tonic supraspinal excitatory influence.

Experimental evidence of a ganglionic action of sympathetic nerves has been published. Recently, Norberg (1964), who used the fluorescent microscopy technique, demonstrated the existence of adrenergic synapses in the parasympathetic, intramural ganglia of the intestinal wall. Kewenter (1965), who used a physiological technique, obtained results suggesting a similar arrangement.

As for the gastric smooth muscle, the vagus nerves can elicit two different responses, one group of efferent vagal fibres produces increased motility, another group elicits a profound relaxation of the corpus and fundus (Jansson and Martinson 1965). The latter group of vagal fibres, which are not adrenergic, elicits a far more powerful relaxation than the sympathetic fibres, at least when they exert their influence on the basal activity in the stomach (Martinson 1965). The present investigation was undertaken in order to study the interaction between the efferent vagal fibres and the sympathetic fibres with regard to their effects on the gastric smooth muscle and with regard to the possible ganglionic site of action of the sympathetic outflow, as suggested for the intestines (Norberg 1964, Kewenter 1965).

Material and Methods

Experiments were performed on 42 cats weighing between 1.6 and 4.4 kg. The animals were deprived of food for 24–36 hours before use. They were anesthetized by i.v. injection of chloralose (70 mg/kg b.w.) after a brief induction narcosis with ether. Free air passage was secured by inserting a tracheal cannula.

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to a 1/4 in. bore plastic catheter connected to a vertical tube which served as a water manometer to measure the pressure that could be applied to the jejunal loop with a syringe filled with isotonic saline. In 15 expts. the nerve bundles along the superior mesenteric artery were cut and electrodes for stimulation were placed on their central ends. The left splanchnic nerves were used for sympathetic stimulation.

The peripheral ends of nerves to be stimulated were placed in ring-shaped bipolar silver electrodes. The poles were embedded in a perspex material insulating the outside of the electrodes from surrounding tissues. The electrodes were connected to a square wave pulse generator (Grass model S4 Stimulator). Pulse intensity, duration and frequency could be adjusted within wide limits.

In some animals the common carotid arteries were dissected free and arranged so as to be easily clamped. In these animals the brachial plexus on one side was also prepared for afferent stimulation in the same way as the other nerves.

polyethylene catheter introduced into the splen

polyethylene catheter introduced into the spinal artery was retrograde direction. Injections of guanethidine (Ismel n^o 118A) and atropine sulphate were given i.v. In some experiments spinal anaesthesia was given at the lower thoracic level. A thin polyethylene catheter was then inserted through a lumbar laminae on a C7 subdural administration 1–1.5 ml of 2% lidocaine solution (Xylocain^o Astra) was usually sufficient to secure adequate blocking. Usually a large volume of dextrane Tyrode solution (10–30 ml) had to be given to control the otherwise extensive fall in blood pressure following spinal anaesthesia of this type.

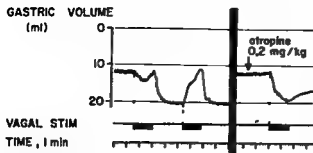


Fig. 1. Cat 3.2 kg. General appearance of gastric motor responses to vagal stimulation as recorded with volumetric technique. Ventral vagus nerve trunk stimulated below the diaphragm 8 imp/sec 2 msec 5 volts. Note that atropine had no effect on basal tone.

Results

Recordings of the gastric volume at constant, low intraluminal pressure in the chloralose anesthetized and vagotomized cat showed characteristic features (Fig. 1). In most cases the volume was initially small and the kymograph record was smooth with rhythmic respiratory movements of almost constant amplitude. By administration of atropine (0.1–1 mg/kg) it was shown that the evidently considerable gastric tone was only slightly or not at all dependent on the parasympathetic excitatory nervous influence (Fig. 1 right). This gastric tone is therefore hereafter called the basal myogenic tone. Application of a vagal stimulation, strong enough to activate both the excitatory and the high threshold 'relaxatory' vagal fibres, produced characteristic responses (cf. Jansson and Martinson 1965). When the initial volume was small, the immediate gastric effect was a relaxation (i.e., an increase of volume), but when the volume was initially fairly large (low initial tone), it produced an initial contraction (Fig. 1 left). During stimulation irregular waves of activity in the stomach wall were superimposed on the rhythmic respiratory movements visible in the resting state. Upon cessation of the stimulation the stomach rapidly relaxed and when the initial volume was small, this after relaxation considerably increased the volume of the stomach which afterwards slowly recovered pre stimulation size. When atropine had blocked the excitatory vagal fibres, the stomach relaxed immediately upon vagal stimulation, provided that the voltage was high enough to activate also the vagal relaxatory fibres (Fig. 1 right).

Stimulation of the splanchnic nerves caused gastric relaxation that was quite small and slow in onset, not being considerable until 'supraphysiological' rates of stimulation were applied (Martinson 1965). Moreover, low rates of splanchnic stimulation sometimes produced excitatory effects (Fig. 2). The relaxations produced by splanchnic stimulation were essentially the same in magnitude whether the basal 'myogenic' tone of the stomach was initially high or low. But when the splanchnic stimulation was performed during continuous stimulation of the vagal excitatory fibres, the inhibition produced by the direct activation of the sympathetic fibres sometimes differed in character. Fig. 2 illustrates such an experiment. Without vagal stimulation graded splanchnic stimulations induced slight excitatory responses at 1 and 4 imp/sec and small and slow inhibitions at higher frequencies. With con-

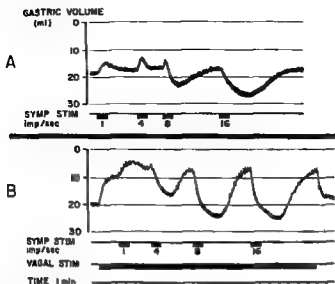
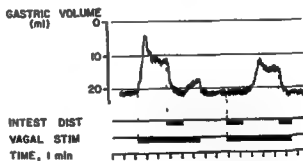


Fig 2 Cat 19 kg Effect of stimulation of the left major splanchnic nerve (3 msec 5 volts) on gastric motility before (A) and during (B) vagal stimulation 8 imp/sec 3 msec 5 volts Note the more rapid and potent inhibitory responses to splanchnic stimulation in B Note also the reversal of response to 4 imp/sec in B

Fig 3 Cat 19 kg Low basal tone after preceding vagal stimulation Clear cut excitatory responses are elicited by vagal stimulation (1 imp/sec 2 msec 5 volts) and these responses are effectively inhibited by intestinal distension To the right constant intestinal distension completely prohibits the vagal response



Continuous vagal stimulation graded splanchnic stimulation elicited prompt and considerable gastric relaxation with the exception of stimulation at 1 imp/sec. These inhibitions induced by direct sympathetic stimulation were so extensive as to suggest that the sympathetic fibres had suppressed the superimposed smooth muscle tone created by the vagal excitatory fibres. The inhibitions were however not obtained so regularly as the inhibitory reflex responses described below.

Distension of an intestinal loop is known to produce a rapid reflex inhibition of intestinal motility (cf Johansson *et al* 1963). But a similar intestinal distension procedure had no influence on the basal myogenic tone of the stomach (Fig 4). However, intestinal distension during continuous activation of the vagal activity

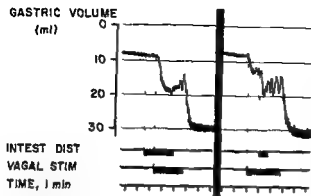


Fig 4 Cat 4.4 kg Appearance of gastric volume response to vagal stimulation and intestinal distension (80 cm H₂O) elicited from a high basal tone level. Note that intestinal distension had no effect on gastric tone when vagus is not stimulated. Vagal stimulation 4 imp/sec 2 msec 5 volts

Fig 5 Cat 3.5 kg Comparison between the effect of intestinal distension (80 cm H₂O) upon the contraction of the stomach elicited by intra arterial infusion of acetylcholine and by vagal stimulation (1 imp/sec 2 msec, 5 volts) respectively

fibres regularly inhibited this vagal excitatory influence promptly and often completely (Fig 3 and 5). When the basal tone of the stomach had been lowered by a preceding stimulation of the vagal relaxatory fibres, clear-cut motor response could be elicited upon activation of the vagal excitatory fibres. This type of vagal response could be reflexly inhibited by subsequent intestinal distension (Fig 3) and it could be completely prohibited by concomitant intestinal distension (Fig 3 right). When the basal myogenic tone of the stomach was initially high *i.e.*, when the stomach contained a small volume (Fig 4) the appearance of the vagal and reflex responses was different. Intestinal distension had no effect on basal tone (left hand panel) but the gastric relaxation on vagal stimulation during continuous distension (left panel) was more marked than it was when no distension was applied (right panel). Rapid inhibition could, however, be produced by intestinal distension during the vagal stimulation also in this case (right hand panel). The same type of

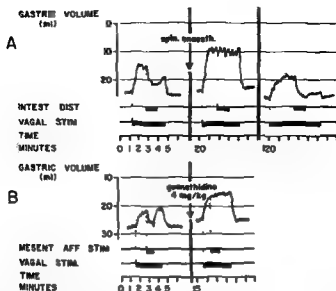


Fig 6 Effects of spinal anesthesia and antadrenergic drug (guanethidine) respectively on the intestino-gastric inhibitory reflex

A Cat 2.6 kg Cervical vagal stimulation 2 imp/sec 2 msec 5 volts intestinal distension 50 cm H_2O Note increased response to vagal stimulation during spinal anesthesia and recovery of response

B Cat 2.3 kg Intra abdominal vagal stimulation 4 imp/sec 2 msec 5 volts Mesenteric afferent stimulation 10 imp/sec 4 msec 3 volts

sympathetically mediated, spinal inhibitory reflex could also be elicited by electrical stimulation of afferent mesenteric nerve fibres (see e.g. Fig 7) and exerted the same influence on the vagal effects on the stomach

On the other hand, as in Kock's (1959) studies on the intestine, inhibition could not be significantly induced by reflexly increased sympathetic discharge during carotid occlusion or during high voltage stimulation of afferent fibres in the brachial plexus. The sympatho inhibitory fibres to the gastrointestinal tract are possibly not involved in this type of reflex sympathetic activation

The fact that intestinal distension had no influence on the basal myogenic tone of the stomach but promptly inhibited vagal excitatory activity suggested a ganglionic site of action of the sympatho inhibitory nerve endings. It was assumed that a non nervous activation of the gastric smooth muscle cells would not be inhibited by the intestino-gastric inhibitory reflex or by direct splanchnic stimulation and it could be shown that the excitatory effect of intra arterial infusion of acetylcholine on gastric smooth muscle was not abolished by activation of the intestino gastric inhibitory reflex (Fig 5)

Spinal anesthesia abolished the inhibitory reflex which recovered as the anesthesia wore off (Fig 6A). — The adrenergic character of the intestino gastric inhibitory reflex was ascertained by studying the response after injection of guanethidine 1 v.

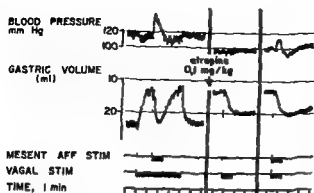


Fig 7 Cat 3.2 kg Left panel Inhibition of vagal cervical stimulation (8 imp/sec 5 msec 5 volts) by stimulation of mesenteric afferents (8 imp/sec 5 msec 5 volts) Atropine 0.1 mg/kg was given between left and middle panels to reveal vagal relaxation response Right panel Vagally elicited relaxation was unchanged by activation of mesenteric afferents as compared with the middle panel of response Note that blood pressure response to afferent mesenteric nerve stimulation is unaffected

A dose of 1–4 mg/kg regularly abolished the intestino gastric reflex inhibition (Fig 6B). The vagal excitatory responses generally augmented during spinal anesthesia and during adrenergic blocking by guanethidine (see Fig 6) suggesting that a sympathetic influence might have been slightly active throughout.

Finally, the action of the intestino gastric inhibitory reflex upon the effects induced by the vagal relaxatory fibres on gastric smooth muscles was also studied. Fig 7 shows an experiment of this type. After atropine, which blocked the vagal excitatory fibres and produced a pure gastric relaxation upon vagal stimulation, the effects of intestinal distension of afferent mesenteric nerve stimulation was studied before and during vagal stimulation. There was now no sign of any sympathetically induced inhibition of the vagal relaxation response.

Discussion

The present results suggest that an adrenergic outflow to the stomach can effectively inhibit vagally elicited excitatory motor responses to the stomach. Procedures such as distension of the intestine or afferent mesenteric nerve stimulation will most effectively activate the sympatho-inhibitory fibres responsible for this adrenergic outflow. The reflex is considered not to be equivalent to the 'enterogastric' reflex of Thomas (1957) as it is elicited from parts of the intestine other than the duodenum and further more seems to resemble the intestino-intestinal inhibitory reflex of Kuntz and Saccomanno (1944). The elicited reflex might then be termed an intestino-gastric inhibitory reflex, and the fact that it was blocked by guanethidine proves that it operates by an adrenergic mechanism. Furthermore, it could be shown that the reflex is dependent on intact spinal connections and that it is not mediated simply via autonomic ganglia, because it is completely abolished by spinal anesthesia. This is in accordance with the findings of Johansson and Langston (1964) concerning the propriospinal intestino-intestinal inhibitory reflex.

The intestino-gastric inhibitory reflex cannot, however, cause relaxation of the gastric smooth muscle cells when their tone is entirely myogenic in origin. The often

considerable basal tone in the stomach preparation, as studied in the present experiments, is evidently myogenic in origin, because it is usually not significantly depressed by atropine, whether it is initially high or low (cf Fig 1 and 7). Only the motor effects induced by the vagal excitatory fibres can be effectively suppressed by the intestino-gastric inhibitory reflex. Intra arterially infused acetylcholine, acting mainly by its muscarinic effect on the smooth muscle cells directly, causes excitatory gastric responses that cannot be inhibited by the reflex. The present results therefore suggest that the influence of the vagal excitatory fibres is blocked by the intestino gastric inhibitory reflex proximal to the neuromuscular junction. Kewenter (1965) arrived at the same conclusion regarding the intestine. Morphological data have proved the existence of adrenergic nerve terminals around ganglion cells of the myenteric and submucous plexa of the intestine (Norberg 1964). The present observations in the stomach agree with an assumption of this type of arrangement and argue against the occurrence of reflex sympathetic inhibition with an action directly on the myogenic activity of the gastric smooth muscles. The smooth muscle cells are evidently only under the direct influence of the high threshold relaxatory vagal fibres distributed only to the corpus fundus region.

Several studies on the central nervous influence on gastric motility have dealt with the problems of gastric motor inhibition (for ref see Elhasson 1960). Since, according to the present study, an underlying activity of the vagal excitatory fibres is a prerequisite for showing the inhibition exerted by the adrenergic nervous outflow, its inhibitory influence can seemingly disappear upon section of the vagus nerves, if such division eliminates a centrally induced vagal tone. If the difference between such a neurogenic tone, mediated via the vagal excitatory fibres, and a purely myogenic tone is not properly considered it might be erroneously concluded from such experiments that centrally induced inhibitory responses are mediated via the vagus nerves, when they are in fact due to sympathetic inhibition of a prevailing activity in vagal excitatory fibres. This might be the case especially in the stomach which in the acutely denervated preparation seems devoid of spontaneous activity in the intramural nerve plexa as discussed above but can nevertheless show a wide variation of myogenic tone.

One would expect efferent splanchnic nerve stimulations to elicit similar inhibition of vagal tone as does the activation of the intestino-gastric inhibitory reflex. Such inhibitions by direct sympathetic stimulation could undoubtedly sometimes be produced, but not in all experiments suggesting that the splanchnic nerves may contain fibres that interfere with the inhibitory adrenergic system which can be so promptly and regularly activated in the intestino gastric reflex.

As pointed out by Lock (1959) with respect to the intestino intestinal inhibitory reflex its efferent link is not activated in connection with the fairly generalized reflex sympathetic excitations produced by e.g. carotid occlusion bleeding or by high voltage afferent somatic nerve stimulation. Conversely it can be concluded that a physiologically elicited reflex vasoconstriction of this type cannot *per se* affect myogenic tone in the stomach or the gap-junc neuromuscular transmission.

of the vagal control of the stomach. Such interferences require blood borne catecholamines.

Under the present experimental conditions the intestino gastric inhibitory reflex system obviously showed some tonic activity, since the vagal responses were augmented by spinal anesthesia and by administration of adrenolytic drugs. Such a tonic reflex activity might be due to activation of peritoneal pain fibres caused by the abdominal surgery. This gives rise to the question whether the reflex system studied might be of physiological significance. The possibility remains that it is a purely pathophysiological event dependent only on pain fibre activation. It can, however, be elicited rapidly and potently also by rather low intraluminal pressures in the intestine — 20 to 80 cm H₂O — which need not to be noxious stimuli. Although the mean pressure within the gut normally is much lower, muscular contractions are able to increase intestinal wall tension considerably when producing so called "type II waves" (see e.g. Davenport 1966, p. 44). The reflex may therefore be involved in some feedback mechanism regulating gastrointestinal motor function (presumably its pressure gradient). It is interesting in this respect to note that the vagal relaxatory nerve fibre system to the stomach is not influenced by the intestino gastric inhibitory reflex system. This also fits in with the assumption that the intestino-gastric inhibitory reflex may be specifically arranged and not merely constitute a diffuse blocking of vagal activity.

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Responses to Linearly Rising Currents in Frog Skeletal Muscle Fibers

By

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Abstract

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... fibers were correlated to the slow active subthreshold processes occurring during stimulation with linearly rising currents

Subthreshold stimulation of skeletal muscle fiber by currents of more than one third of threshold strength gives rise to an active subthreshold response which becomes apparent as a larger depolarization than is to be expected from the passive charging of the cable network of the fiber (Kuffler 1942, Katz 1948) and is also revealed by a conductance change in the membrane (Katz 1942). In a recent investigation (Knutsson 1964) the active and passive subthreshold processes during application of linearly rising currents were extensively studied in frog sartorius muscle and the activation processes were found to vary with the stimulus gradient in a regular manner. Among the results reported were the findings that the active subthreshold potential started at a higher membrane potential value the lower the gradient and that its amplitude increased as the current gradient was lowered.

In a preliminary communication (Knutsson and Skoglund 1963) an account was given of some results obtained in studies of suprathreshold stimulation of frog muscle fibers with currents of different gradients and the present paper will present results from a more comprehensive investigation on spike generation during stimulation with slowly rising currents.

The investigation includes systematic studies of stimulus threshold, firing level and spike configuration during stimulation over a wide range of gradients, and it will be shown that the response patterns of cells with normal resting potential are characterized by a constant firing level and an all or none type of spike response, whereas even a small reduction of the resting potential is associated with a characteristic change into a graded type of response. Attempts were made to relate the different types of response pattern to the preceding subthreshold processes.

Methods

were performed at room temperature (20–22° C)

Two microelectrodes (tip diameters less than $1\ \mu$, resistance 10–20 M Ω , tip junction potential about 5 mV) were inserted 30–50 μ apart into an end plate free zone of an individual muscle fiber. One electrode was used for stimulation with pulses of linearly rising current from a generator delivering "triangular" pulses of varying rate of rise and duration. The output of the generator was connected to the microelectrode via a series-coupled capacitor 0.05 μ F and a resistor 100 M Ω . For measuring the current through the stimulating electrode the voltage drop across a 10,000 Ω resistor in the stimulus circuit was recorded on one of the beams of a double beam oscilloscope after amplification by a d.c. amplifier. In some experiments a constant current delivered from a separate stimulator but applied to the same microelectrode was used to pre-set the membrane potential above or below the resting level. The other microelectrode was used for recording the membrane potential on the other beam of the oscilloscope via a cathode follower with a capacitance neutralizing feedback (Haapainen and Ottoson 1954). Since the propagated response is set up in the immediate vicinity of the stimulating electrode in this type of stimulation, the recorded membrane potential changes correspond closely to the actual potential changes at the point of generation, the inaccuracy introduced by electrotonic spread of slow potential changes between the point of stimulation and the recording point having been estimated to be below 1 mV (Knutsson 1964).

Results

Responses of fibers with normal resting potential

Out of the 300 muscle fibers studied about 95 % had resting potentials of a mean value of -88 mV (range -86 to -90 mV) and their responses to linearly rising currents showed close conformity. The remaining 5 % of the fibers exhibited exceptionally low resting potentials and more varying response patterns as will be illustrated below.

On application of linearly rising stimuli muscle fibers are exposed to current fields of comparatively long duration that may give rise to irreversible membrane changes, and at an early stage of our experiments we found that repeated stimulation might reduce the spike amplitude. To minimize such effects the analyses were based on the first four to eight responses in each fiber.

Figure 1 shows a typical series of responses to linearly rising currents of successively lowered gradients corresponding to spike latencies of up to about 60 msec. Within this latency range the spike potentials take off at about the same membrane potential level, are of similar shape and only moderately reduced in amplitude with lowered gradients. The strength of the stimulating current at the moment of spike initiation has been marked by gaps in the current recordings which show how the threshold

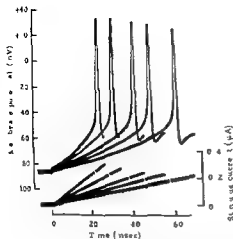


Fig 1

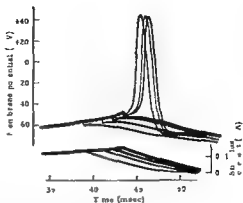


Fig 2

Fig 1 Membrane potential changes in single frog muscle fiber (upper traces) in response to linearly rising currents of different gradients (lower traces). Five superimposed recordings fall of stimulus current and membrane repolarization after break of current not shown. Current thresholds as indicated by gaps in recordings: 0.24, 0.22, 0.20, 0.17 and 0.14 μA . Time from onset of stimulation.

Fig 2 To illustrate determination of the critical membrane potential at an arbitrarily chosen stimulus gradient (3.7 $\mu\text{A}/\text{sec}$). Stepwise increase in duration of linearly rising current. Only final phases of the superimposed stimulus recordings and membrane potential changes are shown and time is given from onset of stimulation. Current fall time 10 msec. small downward artifact in potential recording at break of current. Resting potential -86 mV . Full description in text.

values are reduced as the current gradients are lowered. The results of a close analysis of firing level, stimulus threshold and spike configuration using stimulus gradients over a range from 0.4 to 400 $\mu\text{A}/\text{sec}$ will be presented below.

The rate of rise of the current was calculated from the slope of the current recordings. It is, however, not easy to visualize the velocity of the activation processes on the basis of these measurements.

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In view of the gradual change of the membrane potential into the rising phase of the spike during a slowly rising stimulus it is difficult exactly to determine the level at which the spike takes off, in the present study this has been done by the following method. A current of a given gradient was stepwise increased in strength and the corresponding membrane potential changes recorded as shown in Fig 2. As appears from the superimposed recordings the weakest stimuli applied were below threshold for spike discharge but after a small further increase in current strength a spike was initiated, as also after the subsequent slightly suprathreshold stimuli. By increasing the duration of the linearly rising stimulus by small steps the critical firing potential as well as the current threshold at the chosen gradient could be determined fairly accurately, the margin between a depolarization not inducing and one inducing spike discharge could usually be limited to 1–2 mV. In the experiment

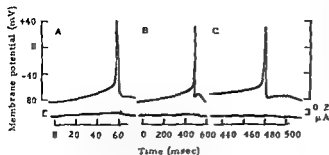


Fig 3 Comparison of responses obtained at two different stimulus gradients resulting in spike discharges at latencies of about 60 msec (A) and 480 msec (B). C same stimulus gradient as in B but recorded at fast sweep speed (same as in 1) showing only final phase of membrane potential change. Time from onset of stimulation.

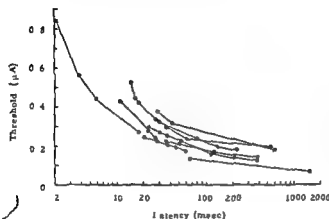
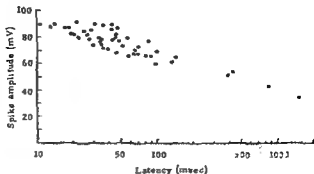


Fig 4 Stimulus thresholds at various linearly rising current gradients plotted against latency of spike response (log scale). Values from each of the nine different fibers tested connected by lines. Resting potentials from -86 to -89 mV.

Fig 2 the critical membrane potential, determined as the membrane potential value at the largest depolarization not resulting in spike discharge, was -50 mV, and determinations made at the same stimulus gradient on eight different fibers of the same muscle gave a mean value of -50 mV (range -48 to -52 mV). This agrees reasonably well with corresponding values previously obtained by square wave stimulation in experiments on frog sartorius muscle (Fatt and Katz 1951, Jenerick 1956). Since this method requires repeated stimulation for each determination of the firing level it was limited to studies of only two different gradients in each fiber and to gradients resulting in latencies less than 300 msec. Experiments performed on twenty fibers showed that over this range of gradients the firing level in a particular fiber varied only within the errors of the method of determination.

The firing level variation at current gradients over a wider range, corresponding to latencies from 10 msec up to about 1 sec, was estimated approximately by photographic projection of the potential curves obtained from single fibers at different stimulus gradients using the same sweep speed (cf Fig 3). Coincident curves at the foot of the spikes were taken as evidence against major changes in firing level. The variations in firing level as estimated by this method should not exceed 5 mV within a range of gradients corresponding to spike latencies up to about 1 sec.

Fig 5 Spike amplitudes at different stimulus gradients plotted against spike latency (log scale). Amplitudes measured from critical firing level to peak of spike. All determinations made in different fibers with resting potentials from -86 to -90 mV.



The stimulus threshold was defined as the amplitude of the linearly rising stimulus current at the moment of spike discharge. That a lowering of the stimulus threshold with decreasing gradient, as illustrated in Fig 1, occurs over a wide range of gradients is shown in Fig 4, in which the stimulus threshold has been plotted against spike latency on a logarithmic scale. Since only a few stimuli were applied to each fiber, nine different fibers were used to cover the whole range of gradients. As also appears from Fig 4, the absolute values of the stimulus threshold at one and the same latency vary in different fibers even though the variations in critical membrane potential are negligible (*cf* above), this is most likely due to variations in the effective resistance of the fibers tested (*cf* Fatt and Katz 1951).

In the experiment illustrated in Fig 1 the stimuli of the two steepest gradients give spikes of equal amplitude, as the gradients are lowered, moderate amplitude reductions occur which however need not be attributed to the difference in stimulus gradient but may also be due to fatigue since these stimuli were the last to be applied in the experiments (*cf* Jenerick 1959). A similar relation between stimulus gradient and spike amplitude was however invariably observed also when starting with the lowest gradient even though the difference in amplitude was usually more pronounced in experiments starting with the steepest gradient.

In one series of experiments only one stimulus was applied to each fiber in order to eliminate fatigue phenomena. The results obtained are shown in Fig 5, in which the spike amplitudes measured from critical membrane potential to peak of spike have been plotted against latencies. Since the spike amplitude varies somewhat with the resting potential, which ranged from -86 to -90 mV in the fibers tested, comparisons of the spike amplitudes at different latencies have to be made with a certain caution. It is evident, however, that a maximum amplitude of 90 mV could be attained as long as the latency was below 40–50 msec and that within this range the variations in spike height were moderate and could in many cases be referred to differences in the resting potentials. At longer latencies on the other hand, the spike amplitude was invariably reduced. The spike duration as measured at the foot of the spike remained unchanged over the range of latency variations tested.

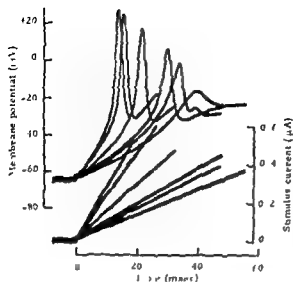


Fig. 6. Superimposed recordings of graded responses obtained at linearly rising currents of different gradients in a depolarized muscle fiber (resting potential -65 mV). \parallel current full and repolarization after current break not shown. Time from onset of stimulus on.

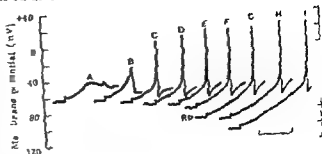
Responses of depolarized fibers

Five per cent of the fibers studied had resting potentials ranging between -60 and -85 mV, i.e. lower than normal, and their responses to linearly rising currents differed from those of the normal fibers. A typical response pattern is shown in Fig. 6. In this fiber (resting potential -65 mV) the spike elicited by the steepest gradient closely resembled that evoked in a normal fiber but as the gradient was lowered there was a pronounced reduction in amplitude and increase in duration of the responses and at the lowest gradient only a 'hump' resulted. At a still lower gradient (not shown in the figure) also this hump disappeared and the curve levelled off. To be able to generate a spike the rate of rise of the current must thus exceed a certain minimal gradient. These characteristic changes in the depolarized fiber occurred at gradients corresponding to spike latencies below 10 msec. in the normal fiber there was no measurable change in spike duration within this range and only a minor amplitude variation. Depolarization can thus be said to convert an all-or-none spike response into a graded response.

As appears from Fig. 6 the transition from slow subthreshold depolarization to spike potential is more gradual than in the normal fibers, and hence it was impossible to define a take off level of the spike. It also proved to be difficult to determine a firing level by the method illustrated in Fig. 2 since the excitability of the depolarized fibers is so unstable that their responses to repeated stimulation tend to be continually varying. For similar reasons accurate determinations of the stimulus threshold were difficult to perform in depolarized fibers.

Comparisons of the response patterns of fibers at different degrees of depolarization suggested that the lower the resting potential the greater was the tendency to increased duration and reduced amplitude of the spike response. In order to study

Fig. 7. Muscle fiber responses to a linearly rising current of constant gradient ($2.8 \mu\text{A sec}^{-1}$) after pre-setting the membrane potential at different levels below (*A-F*) and above (*H-I*) the resting potential (-80 mV , marked *RP*) by polarizing current.



the relationship between the response to a linearly rising current and the membrane potential level a series of experiments was performed in which the membrane potential of normal muscle fibers was pre-set at various levels above and below the resting potential by application of constant current across the membrane. A typical experiment is illustrated in Fig. 7, which shows the responses to stimuli of an arbitrarily chosen gradient, in record *G* at the resting potential of the fiber (-80 mV , marked *RP*), in *H* and *I* after hyperpolarization to two different levels and in *A-F* after varying degrees of depolarization. The initial horizontal part of each recording represents the membrane potential level before onset of the linearly rising current. The only change apparent in *H* and *I* is that a larger depolarization is necessary to attain the critical membrane potential, the spike amplitudes being the same as in *G*. In *A-F* on the other hand the response amplitude is reduced and more so the larger the preceding depolarization, only a "hump" resulting from the current pulse in *A*.

Subthreshold processes and spike activation

Studies of local subthreshold phenomena during square wave stimulation of frog muscle fibers have revealed the presence of two opposing processes (Jenrick 1959). The depolarizing process, which becomes apparent as a deviation in positive direction from the potential change due to passive discharging of the resting membrane is dependent on the presence of extracellular sodium ions and has been attributed to an increase in sodium conductance. In the absence of external sodium there is during sufficiently strong stimulation a deviation from the passive polarization curve in the opposite direction and Jenrick *et al.* (1961) this repolarizing process to an increase in potassium conductance. Other processes may however also be involved e.g. changes of the chloride conductance (Bele and Grayson 1955; Skidgin and Horowitz 1959).

The time dependence of the subthreshold processes was studied by Jenrick (1959) using linearly rising currents of different gradients. The main results of his analysis are schematically summarized in Fig. 8. The tracing refers to the actual membrane potential changes recorded during stimulation at three different gradients and the broken line represents the changes to be expected from a passive membrane polarization. The difference between the actual and the passive mem-

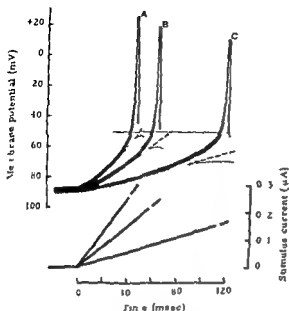


Fig. 8 Membrane potential changes (upper traces A, B and C) induced by three successively lower stimulus gradients (lower traces). Critical firing level indicated by thin horizontal line and current threshold by gaps in stimulus recordings. Broken lines show the passive membrane potential changes to be expected from the current applied across the membrane. The upward deviations of the actual membrane potential from these lines indicate a depolarizing activation which starts at different membrane potential levels in A, B and C. The dotted lines show the typical deviations of the membrane potential in negative direction occurring in fibers when the external solution is deprived of sodium and indicating a repolarizing activation.

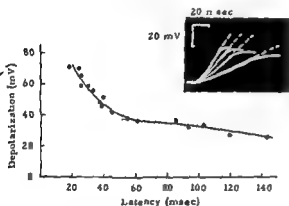


Fig. 9 Muscle fiber in sodium-free solution. Inset: Membrane potential changes obtained at four different gradients in a muscle fiber after replacing external Na by choline. Broken lines represent expected passive membrane potential changes; the points of departure of the actual potential curve from these lines show onset of the repolarizing activation process. Resting potential: 89 mV. Plotted curve: depolarization required to turn on the repolarizing activation process at different stimulus gradients plotted against latency of this activation. Broken line indicates mean depolarization necessary to attain the critical firing level in fibers in normal Ringer's solution.

brane potential changes is due to the depolarizing process and represents the active subthreshold potential which starts at a less depolarized potential level and attains a higher amplitude the lower the stimulus gradient. After replacement of external Na by choline this activation process does not appear, instead the membrane potential deviates in the opposite direction, as schematically shown by the dotted lines in the figure.

The time relations of this repolarizing process were studied more extensively in the present investigation and it was found that this process sets in at a longer latency at all gradients tested than does the depolarizing process, a result consistent with Jenerick's (1959) observation that the repolarizing process has a larger time constant than has the depolarizing process. It could also be established that the repolariz-

ing process, like the active subthreshold potential, starts at a less depolarized membrane potential level the lower the stimulus gradient (cf Fig 8). Illustrative examples of actual curves obtained by stimulation of one and the same muscle fiber at four different gradients are given in the inset of Fig 9. The plotted curve in the figure is based on data from three experiments of the same type representing a wider range of gradients. From this curve it becomes apparent that at the steepest gradients a depolarization of up to 70 mV is required to turn on the repolarizing process and that this process sets in at a lower depolarization the lower the stimulus gradient, a depolarization of 30 mV being sufficient to switch on the repolarizing process at the lowest gradient.

The broken horizontal line in the curve indicates the average depolarization required for spike activation under normal ionic conditions. In this connexion it is noteworthy that the resting potential of the fibers tested in sodium free milieu was about the same (-89 to -90 mV) as in normal ionic milieu. If we presume that the repolarizing process develops in the same manner in fibers in normal and in sodium free milieu, then the curve demonstrates that at steep gradients the repolarizing process will not be switched on until the depolarization exceeds that required for spike initiation whereas at lower gradients, corresponding to spike latencies of more than 60 msec, the repolarizing process will start before the depolarization has reached threshold for spike discharge. Reverting to Fig 8 we will find that in *A* the repolarizing process does not set in until in the course of the ascending phase of the spike, whereas in *B* and *C* it sets in during the subthreshold depolarization. The fact that the spike amplitudes are reduced in *B* and *C* as compared to *A* and more so the more advanced the repolarizing process, favors the concept that the repolarizing process influences the size of the spike.

This concept gains further support from the studies of the corresponding processes in depolarized fibers. The fact that in muscle fibers with normal membrane potential a spike is elicited even at very low gradients, corresponding to latencies of about 1 sec, implies that the depolarizing process is predominant at the critical potential level under normal conditions. In initially depolarized fibers on the other hand no response is obtained when the current rise is slower than a certain minimal gradient; an obvious explanation would be that the repolarizing process then becomes powerful enough to counteract the depolarizing process. A preliminary series of experiments indicates that this is in fact one mechanism behind the behavior of depolarized fibers. Thus, in a series of experiments in which the membrane potential of fibers in sodium free milieu was pre set at different levels and the potential changes compared during stimulation at one and the same stimulus gradient it was found that the repolarizing process sets in at a higher membrane potential value the more depolarized the initial state of the membrane. Such a change of the turning on of the repolarizing process in depolarized fibers should result in an augmentation of the counteraction on the spike depolarization. A predominance of the repolarizing activity when the firing level is attained would explain the absence of spike responses at very low gradients.

Discussion

The membrane responses to linearly rising currents during intracellular stimulation and recording have previously been studied only in motoneurons (Araki and Otani 1959, see also Sasaki and Otani 1961, Frank and Fuortes 1960, Bradley and Somjen 1961). In such experiments the level of displacement of the membrane potential at the moment of spike initiation could not be determined since the stimulus current through the recording electrode caused artifact distortions. Such artifacts are negligible in studies of the muscle fiber which permits the insertion of separate electrodes for stimulation and recording, and isolated muscle is thus a suitable preparation for absolute measurements of the membrane potential changes during application of linearly rising currents.

In muscle fibers with normal resting potential the critical firing level as determined by the exact method of stepwise increasing the stimulus strength did not show any significant variations at gradients corresponding to latencies up to 300 msec. For the lowest gradients tested corresponding to latencies between 300 msec and about 1 sec this method of determination could not be applied (cf. Results above) but comparisons of take off levels indicated that even at those gradients there were no conspicuous changes in the critical firing level. Fatt and Katz (1951) using square wave stimuli of amplitudes just below or just above threshold also found that the level at which the spike originated was independent of the time taken to reach it; their results refer however only to responses of latencies between 0.3 and 8 msec.

The fact that less current strength is required for spike generation the lower the stimulus gradient although the critical membrane potential remains virtually constant may be explained in part by the time constant of the membrane and in part by changes in the subthreshold activation. Owing to the comparatively large time constant of the muscle fiber membrane about 35 msec according to Fatt and Katz (1951) the voltage current ratio across the membrane becomes larger the slower the rise of the current when the gradient exceeds about $0.2 \mu\text{V/sec}$ (Knutsson 1964). Since all gradients used in this investigation were steeper stimuli of a given amplitude were more effective the slower the current rise in other terms to attain the same passive membrane polarization less current strength is required the slower its rise. As illustrated schematically in Fig. 8 a lowering of the stimulus slope results in an increase of the active subthreshold depolarization implying that a larger proportion of the depolarization to the critical firing level is brought about by the activation processes and this would also result in a lowering of the stimulus threshold.

A similar reduction of the stimulus threshold as the rate of current rise is lowered has been observed in spinal motoneurons at short latencies whereas at long latencies the stimulus threshold was found to rise as the rate of rise of the stimulus was lowered (Frank and Fuortes 1960, Bradley and Somjen 1961). In muscle fibers with normal resting potential no such threshold rise could be observed at the gradients used by us but might probably have occurred if still lower stimulus gradients had been applied.

The most striking feature of the muscle membrane properties revealed in the present investigation is the characteristic change in response pattern resulting from even a small decrease of the resting potential. While in normal fibers a lowering of the stimulus gradient has but a moderate effect on the spike height and none at all on its duration, the effect on both these parameters is pronounced in the depolarized cell, the spike being gradually reduced until only a hump like potential occurs just before a minimal gradient is reached at which no spike is generated.

That the spike amplitude is reduced when muscle fibers are depolarized is known from experiments using instantaneously rising currents (Benoit and Coraboeuf 1955). From the present investigation it is evident that when the spikes are elicited by slowly rising currents this amplitude reduction becomes more pronounced and simultaneously the spike duration increases, resulting in a graded response. Thus the time dependent activation processes in the membrane seem to be very susceptible to changes in the membrane polarization.

Similar changes in spike height and duration with depolarization have been observed in spinal motoneurons (Holmodin and Skoglund 1958, 1959) and these neurons resemble muscle fibers also insofar as a minimal gradient could usually not be defined at normal resting potential but was observed in depolarized cells (Bradley and Somjen 1961). In this connexion it is noteworthy that a minimal gradient is usually not found in nerve fibers with intact circulation (Skoglund 1942) but can be demonstrated after interruption of the blood supply (Parrack 1940) as well as in isolated nerve fibers (Tasaki 1950, Diecke 1954) which are liable to depolarize.

On account of the gradual transition from subthreshold potential to spike discharge in depolarized fibers it was not possible to judge whether the critical firing level and threshold in these fibers varied with the stimulus gradient. A gradual rise in threshold is, however, likely to occur as the stimulus gradient approaches the minimal gradient at which excitation is completely abolished. Such a rise in stimulus threshold with lowering of the rate of rise of the current has in fact been observed in toad muscle fibers exhibiting a minimal gradient (Lucas 1967).

The role of the subthreshold repolarizing process as a factor determining the spike amplitude in normal and depolarized muscle fibers has been discussed above (see Results). According to the ionic theory another process influencing the amplitude of the nerve action potential is the inactivation of the sodium-carrying system (Diecke 1954, Vallbo 1964). As far as muscle fibers are concerned no data are available about this process but it is noteworthy that as appears from Fig. 11 the active subthreshold potential preceding the spike is of longer duration and reaches a higher amplitude the lower the stimulus gradient. If this change in subthreshold potential is due to a more pronounced Na^+ activation this may imply that also the Na^+ inactivation becomes larger (cf. Hodgkin and Huxley 1952) which should result in a reduction of the spike amplitude. Considering the complex ionic exchanges that apparently occur in muscle fiber (cf. Adrian and Freygang 1962) and the lack of data on the actual current flow during muscle excitation a correlation of the present results to ionic events can however only be tentative.

Summary

1 The spike responses of frog muscle fiber to linearly rising current were studied using intracellular microelectrodes for stimulation and recording. The rate of rise of the current was varied between 0.4 and 400 $\mu\text{A}/\text{sec}$, resulting in spike latencies ranging from 2 msec up to about 1 sec.

2 In muscle fibers with resting potentials within the normal range (-86 to -90 mV) the critical firing potential was not significantly altered when the stimulus gradient was changed. Direct measurements of the membrane potential at the largest depolarization not resulting in spike discharge gave a mean value of -50 mV (range -48 to -52 mV) and showed no significant variations when made in one and the same cell at various stimulus gradients resulting in spike latencies between 2 and 300 msec. When comparing the take off levels at spike latencies up to about 1 sec the variation in critical firing level was estimated not to exceed 5 mV. The stimulus threshold, i.e. the amplitude of the linearly rising current at spike discharge, decreased with lowering of the gradient.

3 In muscle fibers with low resting potentials (-60 to -85 mV) as well as in normal fibers depolarized to the same values by application of constant current a graded type of spike response was obtained provided that the rate of the stimulus exceeded a certain minimal gradient. These responses were characterized by a pronounced reduction in spike amplitude and increase in spike duration as the stimulus gradient was lowered.

4 A schematic diagram of the subthreshold processes occurring in muscle during stimulation with slowly rising currents, based in part on earlier data is presented and an attempt made to correlate the spike responses in normal and depolarized fibers to these subthreshold processes.

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Uptake, Storage and Release of Histamine by Rat Peritoneal Mast Cells *in vitro*

By

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Abstract

CABUT, M and Ö HAEGERMARK *Uptake, storage and release of histamine by rat peritoneal mast cells in vitro* Acta physiol scand 1966 68 206—214

Rat peritoneal mast cells when incubated in histamine H¹ containing medium were shown to take up and concentrate cellular histamine. The exogenous histamine

ne was taken up
5 HT inhibited

Schayer (1956) showed that rat peritoneal mast cells are able to decarboxylate histidine into histamine, which is then stored in the cells. An important question is whether such cells also are able to take up and store preformed histamine. It has been stated that *in vitro* neoplastic mouse mast cells not only synthesize and store histamine but take up exogenous histamine (Day and Green 1962, Day and Stockbridge 1964). While the present investigation was in progress, Furano and Green (1964) reported that normal rat mast cells also were able to take up extracellular histamine.

In this paper the uptake, binding and release of exogenous histamine by isolated peritoneal mast cells have been studied *in vitro*. The experiments were designed to determine whether histamine is actively or passively taken up by the cells, whether it is then bound to the mast cell granules, and whether it can be released by the histamine liberator compound 48/80.

Methods and Materials

Preparation of cells

Rat peritoneal mast cells were isolated by density gradient centrifugation in Ficoll[®] as described by Uvnäs and Thor (1959). Male rats of the Sprague-Dawley strain weighing 200–400 g were used. A buffered salt solution containing NaCl 154 mM, KCl 2.7 mM, CaCl₂ 0.9 mM, Na₂HPO₄

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40 mM KH_2PO_4 , 2.7 mM, glucose, 1 mg/ml, human serum albumin 1 mg/ml (pH 7.0) was used throughout the experiment i.e. for perfusion of the peritoneal cavity, washing of the cells and as incubation medium. For perfusion the solution also contained heparin 10 IU/ml. After solution and washing procedures the cells were pooled and counted in a Barker chamber.

Incubation technique

The pooled cells were divided into equal aliquots in centrifuge tubes centrifuged at 300 g for 5 min and the supernatants carefully decanted. The cells were then resuspended and incubated at 37°C in 0.5 ml of buffered salt solution to which H^3 labelled histamine had been added. The number of cells in each tube varied in different experiments between 0.3×10^6 and 0.8×10^6 .

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significantly exceed the background

Histamine was extracted from the cells by adding 1.5 ml of 0.1 N HCl and heating in a boiling water bath for 3 min. Aliquots of the extracts were taken for radioactive measurement (histamine assay) and in some experiments for chromatography.

Analysis

Radioactivity was measured in a Tri Carb liquid scintillation spectrometer Model 314 E (Packard Instrument Co.).

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almost identical results when run parallel. All histamine values are expressed as free base.

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and acetyl histamine

Liquid scintillation technique was applied also when measuring the radioactivity of the chromatograms. One centimeter strips of the dried chromatograms were put into the counting vials containing 15 ml of the scintillation fluid. Radioactivity was counted directly without any extraction procedure (Wang and Jones 1959).

Materials

Histamine H^3 d hydrochloride, 10 Ci/mole (Schwarz Bio Research Inc. Orangeburg, N.Y.).

U.S.A.)

Ficoll (Pharmacia)

Results

Table I shows the data obtained from two experimental series where mast cells were incubated with histamine- H^3 1.1 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$ for 60 and 160 min respectively. Histamine uptake amounted to 2.9 and 6.8 $\mu\text{g} \times 10^{-3} \times 10^6$ cells which corresponds respectively to 0.018 and 0.015 per cent of the total histamine content in the cells after incubation. The ratios of intracellular/extracellular histamine H^3 concentrations were 2.8 and 4.7.

TABLE II Effect of enzyme inhibitors on histamine uptake during 60 min. Cells were preincubated with inhibitor for 15 min at 37° C and then histamine H³ was added. Values given as means and standard errors. Figures in parentheses represent number of experiments

Inhibitor	Uptake in presence of inhibitor, as a percentage of uptake in controls
Potassium cyanide, 10 ⁻³ M	89 ± 9 (5)
2,4-dinitrophenol, 10 ⁻³ M	85 ± 34 (3)
Iodoacetic acid 10 ⁻³ M	70 ± 9 (4)
Quabain, 10 ⁻³ M	90 ± 18 (3)

It is evident that the amounts released, 0.2–1.5 µg/10⁶ cells, greatly exceeded the uptake under the corresponding conditions (Fig. 1)

Effect of extracellular histamine concentration on uptake

The histamine uptake was measured after incubation of the cells for 60 min in different concentrations of histamine H³, varying between 4.3×10^{-8} and 1.1 µg/ml (Fig. 3). The uptake was directly proportional to the extracellular histamine concentration.

To ascertain whether the cells could be saturated with histamine they were incubated for 160 min with very high concentrations of histamine. To a constant concentration of histamine H³ (12.5 µCi/ml = 1.4 µg/ml) different amounts of unlabelled histamine were added, the final histamine concentrations varied between 4 µg/ml and 602 µg/ml. The results, corrected for isotope dilution, are shown in Fig. 4. The amounts taken up were directly proportional to the histamine concentrations in the medium except for the highest concentration, where the curve showed a tendency to level off.

Temperature coefficient

Histamine uptake at different extracellular concentrations of histamine H³ was measured at 27° C and 37° C after incubation for 60 min (Fig. 3). Uptake was only slightly decreased when lowering the temperature by 10° C. The temperature coefficients (Q_{10}) for the different histamine concentrations (except for the lowest concentrations) were 1.1, 1.4, 1.4 and 1.1. At the lowest concentration uptake was higher at 27° C than at 37° C. This was probably due to the difficulties in measuring accurately the very small amounts taken up by the cells; the counting rate only slightly exceeded the background.

Effect of enzyme inhibitors on histamine uptake

Table II shows the effect of some enzyme inhibitors on histamine uptake during 60 min. The cells were preincubated with the inhibitor for 15 min and then radioactive histamine, 1.1 µg/ml, was added. In spite of the high inhibitor concentrations used there was only a slight decrease in uptake.

Fig 5 Effect of 5-HT, histidine and 5-HTP on uptake of histamine H^3 $12.5 \mu M$ ($1.4 \mu g/ml$). The cells were incubated at $37^\circ C$ for 160 min. 5-HT (3 expts.) Histidine (3 expts.) 5-HTP (2 expts.) Each point represents the mean uptake as a percentage of uptake in controls. Vertical bars represent range.

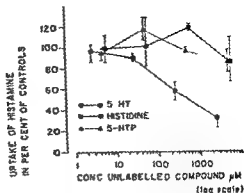
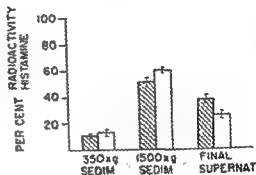


Fig 6 Subcellular distribution of radioactivity \square and of histamine \square in mast cells after incubation with histamine H^3 . The columns represent the means of 7 expts. expressed as the percentage of total radioactivity and total histamine present in the cells. Vertical bars represent standard errors.



Influence of 5-HT, histidine, and 5-HTP on the uptake of labelled histamine

Rat mast cells contain also 5-hydroxytryptamine (5-HT) (Benditt *et al* 1955) which can be synthesized in the mast cell by decarboxylation of 5-hydroxytryptophan (5-HTP) (Hagen and Lee 1958, Lagunoff and Benditt 1959). Moreover, extracellular 5-HT has been shown to enter rat mast cells *in vitro* (Furano and Green 1964). To determine whether the presence of either these compounds or of histidine would influence the uptake of histamine, mast cells were incubated for 160 min with different amounts of unlabelled 5-HT, L-5-HTP and L-histidine in addition to the histamine H^3 ($12.5 \mu M = 1.4 \mu g/ml$). When unlabelled 5-HT, in a final concentration of 250 or 2,500 μM , was added to the incubation medium the uptake of histamine H^3 decreased (Fig 5) whereas the addition of the amino acids histidine or 5-HTP did not affect histamine uptake.

Intracellular localization

After incubation in radioactive histamine the mast cells were washed 3 times, resuspended in 0.34 M sucrose and disintegrated by sonic oscillation at $10 \times 10^4 c/s$ for 15–30 sec (Lagunoff and Benditt 1963). By differential centrifugation at $350 \times g$ for 3 min and $1,500 \times g$ for 45 min, three fractions were obtained: one con-

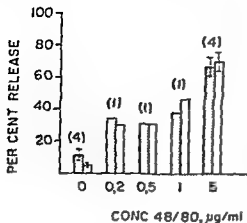


Fig 7 Release of radioactivity and histamine from mast cells (preincubated in histamine H^3) induced by compound 48/80. Each column represents the mean release of radioactivity (hatched bar) and histamine (open bar) expressed as the percentage of total radioactivity and total histamine present in the cells. The figures in parentheses denote the number of experiments. Vertical bars represent standard errors.

cell debris, coarse material and the largest granules (350 g sediment), another containing primarily mast cell granules (1,500 g sediment) and a third fraction (final supernatant) containing soluble material and the remaining subcellular components. The distribution of radioactive histamine and of total histamine (endogenous + exogenous) was determined for the different fractions (Fig 6). Since the amount of exogenous histamine was very small in comparison with the endogenous, the amount of total histamine can be considered approximately equal to that of endogenous histamine. Thus, the distribution of exogenous and endogenous histamine in the cellular fractions was about the same.

1. Histamine release by compound 48/80

Mast cells were incubated for 60 min at 37°C using 1.1 µg histamine H^3 per ml. After washing 3 times the cells were reincubated with the histamine liberator compound 48/80 5 µg/ml. In one experiment different concentrations of 48/80 were used (0.2, 0.5, 1.0 and 5.0 µg/ml). After 10 min the incubation was stopped by centrifugation for 3 min at 350 \times g. Histamine released by 48/80 was recovered in the supernatant, whereas the histamine remaining in the cell residue was extracted from the sediment by heating in a boiling water bath for 3 min in 0.1 N HCl. After neutralizing with NaOH, total histamine and radioactive histamine were determined in the supernatant and the sediment. Radioactive histamine was released in the same relative amounts as the total histamine of the cells (Fig 7).

Discussion

It has been shown in the present investigation that isolated rat peritoneal mast cells *in vitro* are able to take up small amounts of histamine from the surrounding medium.

The histamine uptake is apparently a passive process. This conclusion is based on the following facts. The histamine uptake was small and directly proportional to the extracellular histamine concentration. Saturation was not obtained even

for very high extracellular histamine concentrations. The temperature coefficients were low and metabolic inhibitors did not seem to influence the histamine uptake. This is in agreement with the observations made on neoplastic mouse mast cells, where the histamine uptake was regarded as being passive (Day and Stockbridge 1964).

In our studies with sonically disintegrated mast cells, 55–60 per cent of the endogenous and the exogenous histamine were recovered in the granular fraction. Lagunoff *et al.* (1964) reported a similar distribution using the same technique. However, in intact mast cells all the endogenous histamine is probably stored in the granules. Thus the extragranular histamine observed in the present experiments may be released during the cell disintegration and fractionation procedures. Since the exogenous histamine taken up and the endogenous were bound to the granules in about the same proportion, it seems reasonable to conclude that all the exogenous histamine taken up was also stored in the granules of the intact cells. This conclusion may be supported by the finding that the histamine liberator compound 48/80 which is known to cause extrusion of mast cell granules (Paton 1957) released both exogenous and endogenous histamine in the same relative amounts.

In the present experiments the intracellular/extracellular histamine H^3 concentration ratio was higher than 1, indicating that the concentration of radioactive histamine was higher inside the cell than outside. Furano and Green (1964) observed a concentration ratio of 9.2 after incubation of rat mast cells for 60 min in histamine C^3 , 0.74 $\mu\text{g/ml}$. In the present study the ratio was 2.8 after incubation for 60 min in histamine H^3 , 1.1 $\mu\text{g/ml}$. In the previous section it was concluded that the exogenous histamine taken up was mainly bound to the granules. This implies that the concentration of free histamine in the cytoplasm was low. Thus histamine need not necessarily be transported into the cell against a concentration gradient although the concentration ratio $\approx > 1$ but a passive downhill diffusion of histamine through the cell membrane into the cytoplasm is quite possible. As soon as the histamine has entered the cell it may be rapidly bound to the granules.

Like histamine, 5-HT is bound to the granular fraction of rat peritoneal mast cells (Haegermark unpublished) and in neoplastic mouse mast cells (Hagen Barnett and Lee 1959). The two cell types are capable of taking up 5-HT *in vitro* (Furano and Green 1964; Day and Green 1962). It is not known if 5-HT is bound in the same way as histamine. Day and Stockbridge (1964) found that equimolar concentrations of 5-HT inhibited the uptake of histamine by neoplastic mouse mast cells. In the present investigation no inhibition was observed when the molar ratio between histamine and 5-HT was 1:2 whereas the histamine uptake was decreased to about 60 per cent when the molar ratio was 1:20 and to 30 per cent at the ratio 1:200. It is not possible to make a final conclusion as to the nature of the inhibition. It might be due to cellular injury induced by the high concentrations of 5-HT. Contrary to this, however, are the findings that the cells are still capable of taking up histamine and decarboxylating it into histamine in the presence of these high doses of 5-HT (Cabot and Haegermark to be published). Another possibility may be a competitive inhibition between histamine and 5-HT. Since the present data indicate that histamine

mine passively diffused into the cell, 5 HT should not competitively inhibit the passage of histamine through the cell membrane. On the other hand, if exogenous histamine and 5 HT compete for the same binding sites in the cells, the histamine uptake would be inhibited by an uptake of 5 HT.

In these studies, the presence of extracellular histidine did not influence histamine uptake. When studying the uptake of the amino acid histidine H^3 by mast cells (Cabut and Haegermarck, to be published) it was observed that histidine uptake was not inhibited by the presence of histamine in the medium. These findings are consistent with the idea that there are different uptake mechanisms for histamine and histidine and/or different storage sites for exogenous and endogenous histamine.

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Regulation of Body Temperature and Heat Dissipation at Different Levels of Energy- and Heat Production in Man

By

BODIL NIELSEN

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Abstract

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In treadmill experiments the ratio between total heat production and metabolic energy production was varied by having a subject walking or running uphill horizontally or downhill at different speeds between 2 and 14 km/hr. In the steady state of work of different intensities the body temperature (esophageal or rectal) was found to increase to levels dependent on the energy production but independent of the total heat production. At constant environmental temperature the sweat rate and the skin circulation expressed as conductance of the peripheral tissues varied in proportion to the total heat production and seemed to be independent of the internal temperature. It is concluded that the thermoregulation during work serves two purposes: 1) the setting of the internal temperature at higher levels proportional to the oxygen uptake and 2) the regulation of the heat dissipation in such way that it equals the total heat production independent of the internal temperature level.

In recent experiments (B. Nielsen and M. Nielsen 1965 b) it was found that the thermoregulatory responses produced by active heating, i.e. exercise on a Krogh bicycle ergometer, and by passive heating, through diathermia, were remarkably similar. At the same rate of heat production (or of heat induction), and the same skin and rectal temperatures, the skin blood flow increased to the same level and the increase in sweat rates were not much different. The rectal temperature increased to about the same value and was in both conditions nearly independent of environmental temperature. These experiments therefore seemed to show that the main stimulus for the increase in heat dissipation during the steady state of work is the increased internal temperature caused by the increased heat production and that special "work factors" only to a minor extent influence sweat rate and skin blood flow.

Unfortunately thermoelectric measurements of temperatures were not possible during diathermia. The rectal temperature was determined by a mercury thermometer and was, therefore, possibly not as reliable as the thermoelectric measurements

in the work experiments. The experiments further suffered in that the measurements of temperature could only be performed during small interruptions in the diathermia.

The aim of the present study is to examine more closely the effect of a passive heating at higher rates of heat production and heat induction than could be obtained in the diathermia experiments. The thermoregulatory responses were studied in conditions where the metabolic energy production and sum of the heat production and heat induction could be varied independently, i.e. during grade walking on a treadmill.

Methods

The rectal temperature and the esophageal temperature were measured thermoelectrically, the rectal temperature in four depths and the esophageal temperature just above the diaphragm as described earlier (B. Nielsen and M. Nielsen 1962). The measurements are considered to be accurate to about $\pm 0.025^\circ\text{C}$. The "steady state" values in the graphs are the average of the three measurements in the last 10 min of the experimental period.

1937)

The respiratory gas exchange was measured by the Douglas bag method and samples of the gas were analysed in duplicate on the Scholander apparatus (Scholander 1947). The average of two determinations of gas exchange was used for calculating the energy production in each experiment.

The heat production during the experiments was calculated according to the formula

$$\dot{H} = \dot{M} \pm \dot{W}$$

where \dot{H} is the rate of total heat production in kcal/hr, \dot{M} is the rate of energy production calculated from O_2 uptake (4.9 kcal per l oxygen) and \dot{W} is the external work converted to heat in kcal/hr (Snellen 1960).

\dot{W} was calculated as $G \cdot v \cdot \sin \alpha \cdot \frac{1}{427}$ kcal/hr where

G = body weight in kg

v = speed of walking (running) m/hr

α = inclination of the treadmill in geometrical degrees.

Procedure

the placing of the thermocouples or running at the fixed speed and were made at intervals of about

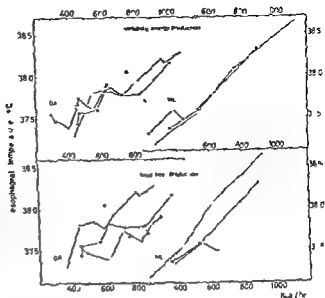


Fig 1 Esophageal temperature in the steady state of work in relation to the rate of metabolic energy production (above) and in relation to the rate of total heat production (below). Environmental temperature 21.5° . Two subjects OA and ML.

Inclination of the treadmill { 0° (horizontal) \bullet — \bullet
 -10° (downhill) \circ — \circ
 $+10^{\circ}$ (uphill) $+$ — $+$

In two experiments the inclination was $+15^{\circ}$ \blacktriangle and -15° \triangle

5 min. After a preliminary period of 20 min of work he stepped off the treadmill and sat down on the seat of the Krogh balance for the first weighing. This pause lasted $1\frac{1}{2}$ –2 min. The subject returned to the treadmill and continued the work in the 45 min experimental period. During the experimental period readings of rectal and esophageal temperatures were taken at 5 min intervals, and skin temperatures and room temperatures were measured 4 times. Expired air was collected in Douglas bags after 25 min and 35 min work in the experimental period. The pulse rate was counted at regular intervals.

After 45 min the work was stopped and the subject was weighed again as quickly as possible that is less than 1 min after stopping.

Thanks to the preliminary "warming up" period heart rate, skin blood flow, ventilation, O_2 uptake, sweat secretion and esophageal temperature reached a steady state, and skin temperature and rectal temperature came very close to a steady state level before the actual experimental period started. The pause necessary for the weight loss determination, had only a negligible effect on the internal temperature, but since the sweat secretion diminished so rapidly after cessation of work, the weight loss would be slightly underestimated. However, the shortness of the pause as compared to the experimental period made this error minimal and nearly equal in all the experiments.

Results

Fig 1 above shows the relationship between the esophageal temperature in the steady state of work and the rate of metabolic energy production in two subjects OA and ML. Below the esophageal temperature is plotted in relation to the total heat production (metabolic \pm work).

The correlation between the esophageal temperature and the energy metabolism seemed to be the same for uphill, horizontal and downhill walking, while the correla-

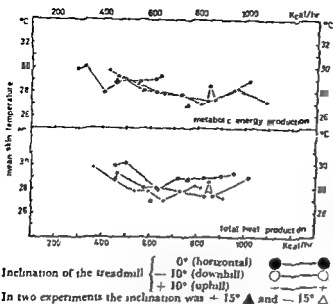


Fig 2 Mean skin temperature in the steady state of work versus metabolic energy production and versus total heat production. Environmental temperature 21.1° . Subject OA

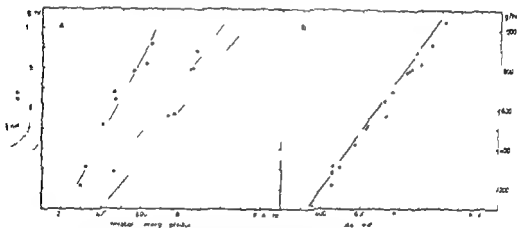


Fig 3 Sweat rate in relation to the rate of metabolic energy production (a) and sweat rate in relation to the rate of total heat production (b). Environmental temperature 21.1° . Subject OA

Inclination of the treadmill $\begin{cases} 0^\circ \text{ (horizontal)} \\ -10^\circ \text{ (downhill)} \\ +10^\circ \text{ (uphill)} \end{cases}$

In two experiments the inclination was $+15^\circ$ \blacktriangle and -15° \triangle

non between the esophageal temperature and the total heat production was different for the three conditions

All the experiments were performed at an average ambient temperature of 21.1° C (average of walls and air). The skin temperature was decreasing only about $1-2^\circ$ C with increasing energy metabolism (Fig 2)

As shown in Fig 3 b and 4 b the sweat rate increased linearly with increase in total heat production. The relationship between sweat rate and metabolic energy production, Fig 3 a and 4 a, however was different for negative work (downhill

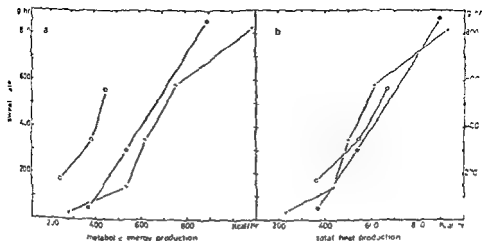


Fig 4 Sweat rate versus metabolic energy production (a) and total heat production (b) for Subject ML. The control subject is represented by filled circles.

Subject ML

1 + 10 (uphill)

● —●—
○ —○—
+ —+—

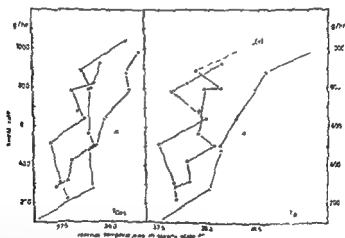


Fig 5 Sweat rate versus esophageal temperature (T_{oes}) and rectal temperature (T_{re}) for Subject OA. The control subject is represented by filled circles.

Inclination of the treadmill { 0° (horizontal) —●—●—
10° (downhill) —○—○—
+ 10° (uphill) + —+—

In two experiments the inclination was + 15° ▲ and - 1° ▼

walking, walking on the level, and positive work (uphill walking). At all times, when metabolic energy production was high, the rate of sweating was high, whether walking downhill, or lower walking uphill, than when walking on the level.

Fig 5 and 6 show the relationship between the sweat rate and body temperature. At the same internal temperature (esophageal or rectal), the rate of sweating was higher when walking uphill than when walking on the level, and higher when walking downhill than when walking on the level.

TABLE I The mean of the steady state values of internal temperatures (T_R and T_{O_2}) and the mean rates of energy production and heat production in three conditions 1) uphill work 2) horizontal work at an energy production equal to the uphill work 3) horizontal work at a total heat production equal to that obtained during uphill work 15 expts Subject ML

Incl of treadmill	Speed km/hr	O ₂ uptake l/min STPD	Energy production kcal/hr	Total heat production kcal/hr	Mean T_{O_2}	Mean T_R
+10	6	2.80	823	675	38.10 SD 0.13 n=6	38.60 SD 0.17 n=6
0°	13	2.82	829	829	37.91 SD 0.08 n=5	38.32 SD 0.11 n=5
0°	11	2.51	680	680	37.85 SD 0.07 n=4	38.28 SD 0.10 n=4

TABLE II The steady state values of esophageal temperature read on graphs like Fig. 1 in three subjects at a rate of energy production of 600 kcal/hr and at a rate of total heat production of 600 kcal/hr. The numbers in parentheses in the table gives the corresponding values of total heat production or energy production respectively

	Incl of treadmill	Esophageal temperature °C		
		Subject OA	Subject OP1	Subject ML
Energy Production 600 kcal/hr	10	37.75 (470)	37.75 (500)	37.70 (480)
	0	37.65 (600)		37.0 (600)
	10	37.80 (900)	37.90 (850)	
Total heat Production 600 kcal/hr	+10	37.85 (775)	38.05 (770)	38.05 (750)
	0	37.65 (600)		37.70 (600)
	10	37.55 (400)	37.60 (400)	37.55 (420)
Number of expts in graph		24	8	12

levels obtained during horizontal running 1) at the same metabolic energy production (823 kcal/hr) and 2) at the same total heat production (ML — external work) (675 kcal/hr). Table I shows that although the rate of total heat production was less during uphill walking the internal temperature was not lower in this condition. The internal temperatures during uphill walking was significantly higher than the values obtained at the same total heat production during horizontal walking (running).

In Table II the esophageal temperatures obtained in steady state of uphill, horizontal and downhill walking are compared at a rate of energy production of

600 kcal/hr and at a rate of total heat production of 600 kcal/hr in three subjects. The values of esophageal temperature were read on graphs relating esophageal temperature and respectively energy production and total heat production, cf Fig 1. In all three subjects the differences between the temperatures reached in the three conditions were largest compared at the same rate of total heat production. The highest values were obtained when the energy production was the highest i.e. during uphill walking. At the same rate of energy production the esophageal temperatures were nearly the same in the three conditions with unsystematic differences.

Discussion

The experiments seem to show that the thermoregulation during work is operating for two purposes. The setting of the internal temperatures at a higher level, and the establishment of an equilibrium between heat production and heat dissipation.

The setting of the internal temperature

The present finding that during work the internal temperatures (T_R and T_{Oes}) increase to new levels, the increases being proportional to the metabolic rates, is in agreement with earlier findings of M. Nielsen (1938), Robinson (1949), B. Nielsen and M. Nielsen (1962) and many others.

In these earlier studies it was established that the level of internal temperature is independent of the ambient temperature within wide limits, but depends on the work intensity.

Normally work intensity, metabolic rate, and heat production vary in proportion to one another and to the changes in internal temperature. However, in our experiments, where the metabolic rate and the total heat production change out of step, it was demonstrated that the work temperature level at a given metabolic rate is independent of the heat production too (Table I and II and Fig 1). This is a direct confirmation of the theory of Nielsen (1938) that the increased temperature during work is not a passive rise, caused by the increased heat production, but is due to a "setting of the body thermostat" at a higher level. The temperature increase would benefit the enzymatic reactions in the working muscles and increase the efficiency of the work (Asmussen and Boje 1945).

This setting of the higher work temperature level in proportion to the energy production must be induced by some factor related to the working condition and, according to our experiments, independent of the heat production. Such work factors have first been considered for the explanation of the regulation of respiration during work. Krogh and Lindhard, (1913) proposed the theory that the work factor was an "irradiation" of impulses from the motor cortex to the respiratory centre. Asmussen, Nielsen and co-workers (1943) found it more likely that afferent impulses from the working muscles were responsible for the increase in ventilation. In a recent work (Asmussen *et al.* 1965), with partial nervous block by curare, they suggested that the mechanism for the increased ventilation during work could be the feedback to the reticular formation of afferent impulses from the muscle spindles.

Similar mechanisms have been considered for the regulation of temperature and sweating. For instance, Kitzing and Bleichert (1965) also used partial neuromuscular blocking with curare in a study of thermoregulation during exercise. However, the increase in motor nerve activity, which presumably must occur during continued work when a certain part of the muscle fibers are blocked by curare, caused no deviation in deep esophageal temperature from pre-infusion values.

In the present treadmill experiments the summated mechanoreceptor output must be much greater during negative work than during positive work at the same energy production because the speed of the movements is about 3–4 times greater during downhill walking (Fig. 8) and the muscle tensions must have been the same. In this case it is unlikely that the thermoregulatory centre obtains information for the temperature setting through neuromuscular reflexes elicited from mechanoreceptors or by irradiation of cortical impulses. However, the adjustment of the temperature level could perhaps be obtained by means of central or peripheral receptors for metabolites occurring in proportion to the metabolic rate.

Asmussen and Nielsen (1947) observed that the rise in rectal temperature during bicycling at a rate of 1100 kpm/min was the same and the O_2 uptake equal at sea level and at a lowered barometric pressure corresponding to an altitude of 4000 m although the pulse rate, ventilation and degree of anaerobiosis was much greater at low pressure. Further, the rise in esophageal temperature during arm work and work with the legs must have been approximately the same at the same O_2 uptake. Estimated from results from Asmussen and Nielsen (1947) and B. Nielsen and M. Nielsen (1962) the degree of anaerobiosis was much higher during arm work which together with the low pressure experiments excludes lactic acid and local H^+ from being the work factor in thermoregulation.

Summing up it seems that the increase of the internal temperatures during work is brought about by some work factor possibly of chemical nature liberated by or in proportion to the extent of the oxydative processes. This induces a change in the setting of the human thermostat perhaps in a manner similar to the action of a pyrogen in fever (Minard and Copman 1963; Cooper *et al.* 1964) although it is not the same substances as is demonstrated by the ineffectiveness of salicylates on the work fever (Dawney and Darling 1962).

The regulation of heat dissipation

The maintenance of the body temperature at any level is possible when equilibrium between heat production and heat dissipation can be established. Therefore when at a certain metabolic rate the rate of heat production is changed by adding or subtracting external energy (walking downhill or uphill) the heat surplus or deficit must be compensated by an equal change in the rate of heat dissipation. Otherwise a temperature equilibrium is not possible.

In this study the environmental temperature was the same in all experiments. The mean skin temperature was almost the same too, decreasing 1–2° at increasing energy production (Fig. 2). The conditions for non evaporative heat loss therefore

are almost the same (Fig. 8). Consequently, when a certain amount of energy is given off to the treadmill (in uphill walking), or induced into the body (during downhill walking), the heat loss through sweating must vary in proportion to the amount of heat produced in the body.

That this actually happens can be seen in Fig. 3a, 4a and 7 below. At the same energy metabolism (and internal temperature, Fig. 1) the sweat rate and skin circulation are much higher during negative work where extra heat is induced into the body than during positive work, where heat is lost as external work. On the other hand, the sweat rates and skin circulations are equal when compared at the same total heat production (Fig. 3b, 4b and 7 above), in spite of different energy metabolism and internal temperature (Fig. 5 and 6). This very nice agreement confirms the finding that passive heating, through diathermia at the same rate of heat production or induction, produced the same sweat rate and skin circulation as active heating.

Recently Benzinger (1959) advanced a theory according to which the rate of sweating is determined by the temperature from a fixed "set point" of the thermosensitive areas in the hypothalamus alone determines the rate of sweating. Hammel *et al.* (1963) proposed an alternative to Benzinger's theory, that the "set point" of the temperature regulating mechanism could change, because they found that the hypothalamic temperature of a dog shivering in the cold could be higher than when it was panting in hot environments. The change in "set point", they suggest, could be produced by impulses from thermoreceptors in the skin, in the core of the body and from other factors, such as state of wakefulness and perhaps also muscular work. Jackson and Hammel (1963) report that the "set" temperature seems to decrease during exercise in the dog and that perhaps also the sensitivity to temperature changes of the thermoregulatory system increases.

In the treadmill experiments the esophageal temperature is the same at the same energy production in the steady state of uphill, horizontal and downhill work (Fig. 1). Also the average skin temperature was nearly equal in all the experiments (Fig. 2), so that the nervous input from the skin receptors must have been almost the same in the 3 conditions. The esophageal temperature is considered a good index of the arterial blood temperature (Cooper and Kenyon 1937). It is thus hard to believe that the hypothalamic temperature should differ under these circumstances, and that the hypothalamic or skin temperatures should be responsible for the differences in sweat rate and skin circulation found when the rate of heat productions were different.

Robinson (1962, 1963) and Robinson and co-workers (1963) suggested that neuromuscular reflexes take part in the regulation of sweating and skin circulation during work, but they found it less likely that these reflexes were elicited from mechanoreceptors, and assumed that thermoreceptors, in the muscles were a more likely source.

It would be interesting to measure muscle temperature during positive and negative work to see if the local temperature were higher during negative work even when the rectal temperatures were equal (Fig. 5 and 6), thus providing the extra stimulus for the sweating mechanism and the skin circulation.

Similar mechanisms have been considered for the regulation of temperature and sweating. For instance, Hitzing and Benschert (1955) also used partial neuromuscular blocking with curare in a study of thermoregulation during exercise. However, the increase in motor nerve activity, which presumably must occur during continued work when a certain part of the muscle fibres are blocked by curare, caused no deviation in deep esophageal temperature from pre-infusion values.

In the present treadmill experiments the summated median receptor output must be much greater during negative work than during positive work at the same energy production because the speed of the movements is about 3-4 times greater during downhill walking (Fig. 2) and the muscle tensions must have been the same. In this case it is unlikely that the thermoregulatory centre obtains information for the temperature setting through neuromuscular reflexes elicited from median receptors or by irradiation of cortical impulses. However, the adjustment of the temperature level could perhaps be obtained by means of central or peripheral receptors for metabolism occurring in proportion to the metabolic rate.

Åstrand and Nielsen (1947) observed that the rise in rectal temperature during bicycling at a rate of 110 kpm/min was the same and the O_2 uptake equal at sea level and at a lowered barometric pressure corresponding to an altitude of 4000 m, although the pulse rate, ventilation and degree of anaerobiosis was much greater at low pressure. Further, the rise in esophageal temperature during arm work and work with the legs must have been approximately the same at the same O_2 uptake, estimated from results from Åstrand and Nielsen (1947) and B. Nielsen and M. Nielsen (1952). The degree of anaerobiosis was much higher during arm work, which together with the low pressure experiments excludes lactic acid and local H^+ from being the "work factor" in thermoregulation.

Summing up it seems that the increase of the internal temperatures during work is brought about by some work factor, probably of chemical nature, liberated in or in proportion to the extent of the oxidative process. This induces a change in the setting of the human thermostat, perhaps in a manner similar to the action of a pyrexin in fever (Milard and Caplan 1963; Cooper et al. 1964), although it is not the same substance as is demonstrated by the ineffectiveness of salicylates on the "work fever" (Dawson and Davies 1962).

The regulation of heat dissipation

The maintenance of the body temperature at an level is possible when equilibrium between heat production and heat dissipation can be established. Therefore, when at a certain metabolic rate the rate of heat production is changed by adding or subtracting external energy, walking downhill or uphill, the heat surplus or deficit must be compensated by an equal change in the rate of heat dissipation. Otherwise a temperature equilibrium is not possible.

In this study the environmental temperature was the same in all experiments. The mean skin temperature was almost the same too, decreasing 1-2 $^{\circ}C$ at the same energy production (Fig. 2). The overall skin temperature has been considered

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Effect of Ethionine on the Absorption of Palmitic Acid- $1-C^{14}$ in the Rat

By

ESKO KARVINEN and MATTI MIETTINEN

Received 30 March 1966

Abstract

KARVINEN, E. and M. MIETTINEN *Effect of ethionine on the absorption of palmitic acid $1-C^{14}$ in the rat* Acta physiol scand 1966 68 228—230

Ethionine administration to female rats resulted in significantly lower rate of absorption of ingested palmitic acid $1-C^{14}$ as compared with that of saline treated controls

It has been implied that ethionine may facilitate fat absorption since increased chylomicron counts were noted in rats with ethionine induced fatty livers (Fidwall 1956). In earlier studies from this laboratory, it has been found that ethionine administration to female rats resulted in an increase in the radioactivity of the chylomicron fraction after ingestion of cholesterol $4-C^{14}$ (Karvinen and Miettinen 1962) but no such increase was noted after ingestion of palmitic acid $1-C^{14}$ (Karvinen *et al* 1964).

In the present paper, studies on the absorption of palmitic acid $1-C^{14}$ in the female rat are reported.

Material and Methods

Female rats of the Wistar strain weighing 200 to 250 g were caged individually and given a standard diet for 3 weeks *ad libitum*. During the experiment the rats were fed with graham flour and water. They were given daily 3 intraperitoneal injections of ethionine or saline at 9 a.m., 3 p.m. and 10 p.m. On the day of the experiment the rats were given daily at 9 and 3 p.m.

TABLE I Radioactivity of the feces and intestine serum lipid and liver fat of the ethionine and saline rats 28 hrs after ingestion of palmitic acid 1 C^{14} (mean \pm standard deviation)

Number		Feces and intestine combined		Feces eliminated counts per 100 sec	Lipid from 1 ml of serum counts per 100 sec	Liver fat counts per 100 sec
		Counts per 100 sec	% of dose ingested			
Ethionine rats	5	101,724 \pm 12,247	56.2	393 \pm 360	19 \pm 33	4.18
Saline	8	21,229 \pm 14,422	11.7	2,209 \pm 686	62 \pm 32	6.1
t value of difference		10.55		5.98	0.69	
P		0.001		0.001	None	0.00

and the acidified mixture was allowed to stand overnight. Then the lipids were taken up in petroleum ether and washed with 50% ethanol and with water.

The serum lipids were extracted as described earlier (Karvonen *et al.* 1964). Samples of the lipid were then plated on steel planchets and counted. The counts were corrected for mass absorption.

Results

In Table I the radioactivities remaining in the intestine of excreted in the feces are given. It is seen that ethionine administration resulted in a significant reduction in the absorption of labeled palmitic acid from the intestine. Radioactivity of the blood serum lipid was not significantly altered by the ethionine treatment, whereas that of the liver fat was tripled.

Discussion

The present results indicate that the absorption of ingested palmitic acid 1 C^{14} from the intestine is slowed down in the ethionine treated female rat. The experimental animals finished eating the ration containing the labeled fatty acid in 15 min, so it seems unlikely that a slower rate of intake of the labeled material by the ethionine animals would be responsible of the finding.

The data on the radioactivity actually eliminated in the feces (Table I) suggest that the rate of propulsion of the gastro-intestinal contents in the ethionine-treated rats may be slightly slower than that of the saline controls. Differing rates of propulsion could eventually lead to differing rates of absorption. Since the process of fat absorption is completed already at the lower end of the small intestine and since some of the labeled fatty acid had already passed with the feces, it seems rather unlikely that a slower rate of propulsion in the gut of the ethionine rats would be the main cause of the slower absorption obtained in these animals.

It is known that ethionine administration leads to acinar cell necrosis in the rat pancreas (Herman and Fitzgerald 1962) resulting in lowered pancreatic lipase

activities in the intestine (Libre and McFarland 1964) The pancreatic juice is rather important in the utilization of fat It has been shown that exclusion of pancreatic juice results in a decrease in the absorption of tripalmitin from rat's intestine, even though no significant change in the absorption of palmitic acid could be demonstrated in pancreatic duct-ligated rats (Karvinen *et al* 1957)

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Collagen Components in the Consecutive Extracts of Rat Skin

By

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Abstract

HEIKKINEN, E and E KULONEN *Collagen components in the consecutive extracts of rat skin*
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The present results demonstrate that the various fractions of skin collagen are very different and that the solubility is a matter of definition. The methods of various authors for the preparation of the soluble fractions of collagen vary greatly. Standard procedures and a more defined terminology are urgently needed.

The purpose of this note is to draw attention to the quite different component patterns in the collagen fractions which have been obtained with various solvents.

That fraction of collagen which is soluble in dilute solutions of neutral salts is considered to be the youngest (Jackson 1957). It is assumed that collagen is continuously converted to less soluble and more mature forms by the formation of intra and intermolecular cross links and that in growing connective tissue there is a continuous spectrum of various aggregates of different ages (Jackson 1960, Heikkinen *et al* 1964a, b). By the present methods it is possible to test the properties of collagen solutions in the terms of the α , β and γ (or x)-components (Nanto *et al* 1964) and consequently of the intra and intermolecular cross links.

Various collagen fractions were obtained from skins of young rats (25—30 g) according to the flow sheet below where the amounts of each fraction are indicated. Only 11.3 per cent of total collagen remained in the final insoluble fraction. In Fig. 1 the starch gel electrophoretic patterns of the components of denatured collagen fractions are presented. Collagen which is extractable with dilute salt solution consists mainly of non linked α -components. When the ionic strength of the solvent is increased additional collagen is dissolved and it contains also β -components with intramolecular cross links. In acetic acid collagen swells and a great part of the residue dissolves. This fraction is characterized by a dominance of β components and of larger aggregates, the x (or γ , δ) components. Denatur

TABLE 1 Flow sheet of the extraction and purification of different collagen fractions from rat skin. If not stated otherwise the manipulations were carried out at $+4^{\circ}\text{C}$ and the centrifugations with refrigerated equipment for 60 min at $35\,000\times g$.

Rat skin

was homogenized in 0.15 M NaCl (2.5% wet weight) with Sorvall homogenizer for 2 min. After 24 hr the mixture was centrifuged and the extraction was repeated four times.

Residue was extracted four times with 0.30 M NaCl and centrifuged		Combined supernatants were filtered and dialyzed against 0.1 M citrate buffer, pH 3.6. Collagen was precipitated with sodium chloride (final concn 15%) collected by centrifugation and dissolved in 0.1 M acetic acid. This procedure was repeated twice. Collagen was finally precipitated by dialysis against several changes of 0.01 M disodium phosphate and water, collected by centrifugation, dissolved in 0.1 M acetic acid and lyophilized. The yield of 0.15 M NaCl-soluble fraction was 11.3% of total collagen.
Residue was extracted four times with 0.45 M NaCl and centrifuged	Combined supernatants were handled as the 0.15 M supernatant. The yield of 0.30-M NaCl-soluble fraction was 21.4% of total collagen.	
Residue was extracted four times with 0.5 M acetic acid and cen- trifuged	Combined supernatants were handled as the 0.15 M supernatant. The yield of 0.45 M NaCl-soluble fraction was 18.2% of total collagen.	
Residue was extracted once with 0.5 M acetic acid at -40°C for 30 min and centrifuged		Combined supernatants were purified by precipitation with NaCl (final concn 10%) and subsequently by dialysis against several changes of 0.01 M disodium phosphate and water. Collagen was collected by cen- trifugation, dissolved in 0.5 M acetic acid and lyophilized. The yield of acetic acid-soluble fraction was 29.4% of total collagen.
Residue was lyophilized. The yield of the insoluble fraction was 11.3% of total collagen.	Supernatant was lyophilized. The yield of warm acetic acid-extractable fraction was 8.4% of total collagen.	

at rather low temperature (at -40°C for 30 min) yields a further soluble fraction containing proportionally more of these larger fragments (cf. Pikkariainen *et al.* 1964) with intermolecular bonds. The last insoluble residue may be solubilized to collagen components by digestion with pepsin. Other work (Lampiaho *et al.* 1965) shows that fractions of different metabolic ages can be demonstrated in conventional preparations of insoluble collagen.

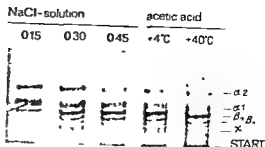


Fig 1 Starch gel electrophoretic patterns of the consecutive collagen fractions from rat skin. The preparations are described in the flow sheet.

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Chloride and Hydrogen Ion Distribution between Human Red Cells and Plasma

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Abstract

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Distribution of Cl⁻ and H⁺ between human red cells and plasma has been studied after variations of plasma pH (induced both by changing pCO₂ and changing bicarbonate concentration). The distribution ratio of chloride $Cl_{cell}/Cl_{plasma} = r(Cl)$ and of hydrogen ions $H_{plasma}/H_{cell} = r(H)$ showed the following relations to plasma pH

$$r(Cl) = 3.319 - (0.339 \text{ pH}_{plasma})$$

$$r(H) = 3.091 - (0.335 \text{ pH}_{plasma})$$

The conception that the distribution of Cl⁻ and H⁺ is a Donnan distribution is supported by the distribution of Cl⁻ and H⁺ found after addition of an impermeant negatively charged ion (citrate) to the extracellular phase. The normally negative potential difference between cells and plasma may be inverted in the presence of citrate. The effects on ion distribution seen when normal cells are suspended in isotonic sucrose or glucose can be explained as being caused by diffusion potentials which arise because of the much higher permeability of the red cell membrane towards anions than towards cations. Calculation of the water shift to be expected as the osmotic consequence of the redistribution of diffusible anions between cells and plasma (3 H₂O/1 pH 423 m H₂O/kg RBC solids) was found to agree with the experimentally determined value of 401 g H₂O/kg RBC solids.

The conception that the distribution of hydrogen and chloride ions between cells and plasma is a Donnan equilibrium was introduced by the pioneering works of Warburg (1922) and of Van Slyke, Wu and McLean (1923). The validity of this theory has been generally agreed upon by many investigators (e.g. Dill, Edwards and Consolazio 1937, Litzsimons and Sendroy 1961, and Bromberg *et al* 1965). However other works have questioned that the Donnan theory can always explain the distribution of hydrogen ions and rapidly diffusing anions between cells and plasma. Peters, Tulin, Danowski and Hald (1947) found that when the distribution of chloride and combined CO₂ between cells and plasma of ovine erythrocytes was altered, by varying the CO₂ tension, the distribution of H⁺ was not altered in isotonic sucrose solution. This was interpreted as evidence that the distribution of H⁺ is not a Donnan equilibrium.

Bubnoff and Riecker (1961) found that hydrogen ion exchanges between cells and plasma after altering bicarbonate concentration took place very slowly, reaching equilibrium after one hour at 37° C. Siggaard Andersen (1961) noted a considerable shift of hydrogen ions from plasma to cells lasting several hours, when blood containing sodium fluoride was stored anaerobically at 38° C. He considered the possibility that the hydrogen ion shift observed was secondary to diffusion potentials created by passive diffusion of Na and K, (since K leaves the cells much faster than Na enters in the presence of fluoride) (Dunker and Passow 1950). However, it was also suggested that H⁺ distribution in normal blood might be maintained by an active mechanism, extruding H⁺ of the cells, and that the hydrogen ion shift observed might be a result of inhibiting the energy supply of such an H⁺ transport with NaF. No matter what is the cause of the transfer of hydrogen ions from plasma to cells in the presence of fluoride, this phenomenon questions the value of the results of Fitzsimons and Sendroy (1961) and of Bubnoff and Riecker (1961). Both these groups carried out experiments on cells in which glycolysis had been arrested by the addition of fluoride.

The possibility that H⁺ is transported actively into the red cell by a hypothetical carrier extruding Na⁺ and accumulating K⁺ and H⁺ has been considered by Ussing (1960 p. 78). Since reasonable doubt has been thrown on the passive distribution of H⁺ between cells and plasma, this study was carried out on cells metabolizing at 38° C, since it might be argued that the separation of cells and plasma at 5—8° C as performed by Bromberg *et al.* (1965) might inhibit an active transport mechanism as efficaciously as the employment of metabolic poisons. It was thus found desirable to reinvestigate the distribution of Cl⁻ and H⁺ between cells and plasma with a technique that can eliminate the objections to previous investigations presented above. In the present work chloride and hydrogen ion distribution of glycolyzing cells are examined in the plasma pH range 6.40 to 8.50. The variations found support the conception that a Donnan equilibrium exists across the red cell membrane with regard to chloride and hydrogen ions. As a test of passive distribution of H⁺ and Cl⁻ it has been examined if the addition of an impermeant polyanion (citrate) to plasma leads to the predicted changes of ion distribution by neutralizing the effect of the non-diffusible anions of the cell. Finally it has been examined whether the water shift between cells and plasma which is observed after acute variations of plasma pH (Funder and Wieth 1966a) may be quantitatively explained as an osmotic consequence of a redistribution of diffusible ions secondary to a change in a Donnan potential between cells and plasma.

Methods

Heparinized blood 10:1 v/v per ml was sampled from healthy donors. Oxygenation and adjustment of CO₂ partial pressure was carried out by recirculating 50–100 ml of blood in a modified oxygenator unit of a heart lung machine (Frederiksen, Ryge and Therkelsen 1967). The following gas mixtures were employed for the adjustment of carbon dioxide partial pressures: 1. 5.6% CO₂, 94.4% atmospheric air; 2. 8.0% CO₂, 20% O₂, 3% atmospheric air; 3. 0.03% CO₂. Complete oxidation of hemoglobin was ascertained by analysis (Sylvén 1953). To study the influence of primary of pCO₂, blood was exposed to the gas mixtures in the sequence: 1. 3.1% N₂, 96.9% O₂.

attain equilibration of the blood with the gas mixtures 2) and 3) Blood was drawn into a 10 ml syringe at random intervals, thereby obtaining a sequence of changes in $p\text{CO}_2$. The syringe was thermostated in a water bath at 38°C and after a 10 minutes temperature equilibration period 8 ml of blood were transferred to CO_2 equilibrated blood.

isolated erythrocytes amounted to 1.1--1.3 per cent (w/w) by the centrifugation employed. The isolated erythrocytes were immediately capped by a tight fitting PVC-cap. Buffy coat was removed.

RBC were immediately lysed by freezing and thawing the tubes thrice in a dry ice-alcohol mixture. After the last thawing the lysed cells were immediately submitted to pH measurement at 38°C .

0.14 M NaOH. Addition of acid or base was performed in the following way. After equilibration with gas mixture 1 (to ensure oxygenation) 10 ml blood samples were spun at 1500 \times g for 5 min. 4 ml of plasma was withdrawn and acid or base added to the plasma. Acid or base treated plasma was thereafter slowly added to the lightly packed cells. After resuspending the red cells, blood was transferred to a 10 ml syringe and incubated at 38°C following the procedure described above. The maximal additions of 0.14 M HCl or NaOH employed were 3 ml and 1.75 ml respectively. This equals the addition of 42 meq acid and 24.5 meq base per litre of blood.

Addition of Sodium Citrate. Oxygenated blood was diluted with varying amounts of a solution of 0.05 M tertiary sodium citrate (0.15 eq Na per litre dissolved in a 2.5% glucose solution). 10 ml of citrate/blood mixtures were produced by mixing various amounts of blood and citrate solution (maximal amount of citrate solution 4.55 ml per 10 ml blood/citrate mixture). After 10 min incubation at 38°C the blood/citrate sample was treated further as described above.

pH measurements were carried out at 38°C with micro electrode equipment (Electrode Radiometer E 5021 with pH-meter 25 SE). The phosphate buffers (pH 6.81 and 7.38 at 38°C) used as standards of reference were in accordance with the NBS-scale (Bates 1934).

Chloride determinations in lysed cells and plasma were carried out by electrometric titration with 0.01 M AgNO_3 (Titrator TTT1a Radiometer with silver electrode P 401 and calomel electrode K 601) with liquid junction of saturated K_2SO_4 . As erythrocyte proteins are known to bind Ag

Terminology of Ion Distribution Ratios. The relation between potential difference (E) and the distribution of a freely diffusible ion in two phases separated by a semipermeable membrane is given by

$$\varphi_o - \varphi_i = E = - \frac{RT}{zF} \ln \frac{[a]_i}{[a]_o} \quad (\text{volt})$$

where the subscripts i and o denote inside and outside phase respectively. φ is the electrical potential, R (8.314 joule $^\circ\text{K}^{-1}$ mole $^{-1}$) is the gas constant, T is the absolute temperature, z is the valency of the ion considered, F Faraday's constant (96493 coulomb eq $^{-1}$), and $[a]$ designates the activity of the ion considered in the two phases indicated by the subscripts i and o . A detailed

concentrations the tacit admission is made that the expression is not strictly valid. The difference, if the ratio of the activity coefficients of chloride ions in cells and plasma is not unity

In text, tables, and figures ion distribution ratios are indicated by r . The ratio of chloride in cells and plasma

$\text{Cl}_{\text{cell}}/\text{Cl}_{\text{plasma}}$ is indicated as r_{Cl} ,

correspondingly the ratio of hydrogen ion activities

$\text{H}^+_{\text{plasma}}/\text{H}^+_{\text{cell}}$ is indicated as r_{H} .

Results

1 Distribution of H^+ and Cl^- as a function of plasma pH

Data of chloride and hydrogen ion distribution between plasma and cell water found at various plasma pH values are given in Table I, and the regression lines of r_{Cl} and of r_{H} versus plasma pH are shown in Fig. 1.

The interdependence between hydrogen ion distribution and the pH of plasma was found to be expressed by the equation

$$r_{\text{H}} = 3.094 (\pm 0.014) - 0.335 (\pm 0.005) (\text{pH}_{\text{plasma}}) \quad (1)$$

The correlation coefficient of this regression line is $-0.995 (\pm 0.015)$.

The regression line of the interdependence between r_{Cl} and pH of plasma was similarly

$$r_{\text{Cl}} = 3.319 (\pm 0.028) - 0.359 (\pm 0.010) (\text{pH}_{\text{plasma}}) \quad (2)$$

The correlation coefficient of the regression line is $-0.993 (\pm 0.026)$.

No differences were found in the ion distributions effected by changes of pCO_2 , compared to the distributions found after addition of HCl or NaOH (viz. Fig. 1).

2 Chloride and Hydrogen Ion Distribution in the Presence of Citrate in the External Medium

It was felt that measurement of ion distributions under conditions, where the Donnan potential could be expected to be changed, or even inverted, would yield valuable information as to the legitimacy of evaluating the membrane potential of the red cell from the distribution of diffusible ions.

It may be anticipated that the potential difference between cells and plasma can be diminished — or even inverted — if non-permeating anions are added to the

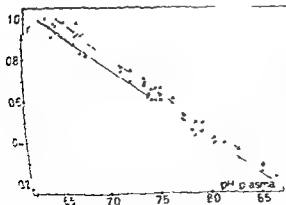


Fig. 1. r_{Cl} (triangles) and r_{H} (circles) as functions of plasma pH. Results indicated by open signatures were obtained after variation of pCO_2 , results indicated by solid signatures after addition of HCl or NaOH. The solid line represents the equation $r_{\text{H}} = 3.094 - 0.335 \text{ pH}_{\text{plasma}}$. The dashed line represents the equation $r_{\text{Cl}} = 3.319 - 0.359 \text{ pH}_{\text{plasma}}$.

TABLE II Hydrogen ion and chloride ion distribution between cells and plasma under conditions where potential difference between cells and plasma is affected. Calculated potential differences agree within 2 mV in spite of the inversion of potential

Citrate conc. medium mM	pH ext. medium	pH lyzed RBC	$r_{(H^+)}$	Cl ext. medium meq/kg H_2O	Cl RBC meq/kg H_2O	$r_{(Cl^-)}$	Osmolality ext. medium	Calculated potential difference mV	
								from $r_{(H^+)}$	from $r_{(Cl^-)}$
92	7.26	7.19	0.85	86.7	68.5	0.79	0.283	-4.4	-6.3
233	7.21	7.20	0.98	61.1	58.6	0.96	0.284	-0.6	-1.1
322	7.14	7.22	1.20	37.9	47.1	1.24	0.286	+4.9	+5.8
0 ¹	7.12	7.16	1.10	36.8	37.6	1.02	0.290	+2.6	+0.5

¹ 5 ml blood + 5 ml 5% glucose

external medium. Since citrate is known to permeate the red cell membrane extremely slowly (if at all), the following experiment was designed.

Heparinized blood was diluted with a citrate solution (isotonic with plasma), containing 150 meq Na⁺/litre and 2.5% (w/v) glucose per litre to accomplish isotonicity with plasma. The results obtained are listed in Table II, which shows the pH of the external medium and lyzed cells, and the chloride concentration of plasma and cells after isolation carried out at 38° C. It appears from the results that the distribution of diffusible ions is affected in the predicted way by the presence of the non diffusible poly-anion in the plasma. Measurement of osmolality of the external medium (by determination of freezing point depression) showed only negligible changes. Potential differences — as calculated by inserting the values found for $r_{(Cl^-)}$ and for $r_{(H^+)}$ into the Nernst equation are shown in the far right columns. It is seen that the calculated potential differences are affected in the same direction and approximately to the same degree, whether they are estimated from hydrogen ion — or from chloride distribution. It further appears that the normal negative P.D. of the red cell under these conditions is inverted to positive values at a citrate concentration of 23 mM = 69 meq citrate per litre external medium.

The positive P.D. values obtained after adding citrate in sufficient concentration to the external medium is quite stable, as may be reasoned from the experiment shown in Fig. 2. This figure shows the recording of pH of the external medium of a blood citrate mixture with a citrate concentration of 32 mM. During an observation period of 1.5 hrs at 38° C. pH of the medium only fell from 7.20 to 7.18. This fall of external pH may be due to continued metabolic activity of the RBC. However, a rise of external pH is to be expected if membrane potential was reverted during the experimental period due to penetration of citrate into the cells.

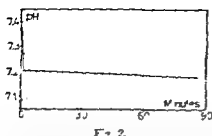


Fig 2 pH of the medium of a suspension of red cells incubated at 38 °C. At zero time 5 ml of a citrate solution containing 50 mM citrate I, 150 meq Na⁺ I and 2.5 w/v % glucose pH 7.40 is added to 5 ml of heparinized blood. Calculated citrate concentration of the extracellular phase 30 mM. After the initial pH change induced by mixing blood and citrate pH of the medium only showed a slight decreasing tendency. pH was measured continuously; readings were made with intervals of 0.5 to 3 min according to rate of pH change. Identical results were obtained in 5 experiments.

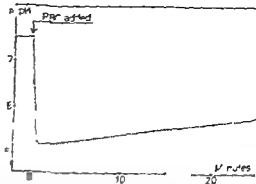


Fig 3 pH of a solution of 5 w/v % glucose after the addition of 300 mg packed red cells to 10 ml glucose solution. The experiment was carried out at 22 °C. pH was measured continuously; readings were made with intervals of 0.5 to 3 min according to rate of change. The figure is representative of a total of 20 experiments.

3) Chloride and Hydrogen Ion Distribution between Cells and 5 per cent Glucose

Fig 3 shows the effect on external pH caused by suspending 300 mg of packed RBC in 10 ml of a 5 w/v % glucose solution pH 7.20 at 22 °C. The rapid initial fall of the environmental pH about 2 pH units — from pH 7.20 to 5.20 — indicates a rapid redistribution of H⁺ and OH⁻ across the membrane induced by the diffusion of anions Cl⁻ and HCO₃⁻ traversing the membrane at a rate which is initially 10⁴ times faster than that of sodium and potassium (Passow 1964). The pH of the RBC employed in the experiment was 7.20.

It may be assumed that the pH of the erythrocyte fluid is virtually unaffected by a calculated liberation of 0.2 meq H⁺ / kg RBC, sufficient to lower pH of the unbuffered medium from 7.20 to 5.20. The maximal potential difference calculated by the Nernst equation from the maximal change of external pH is therefore $58 \times 2 = 116$ mV (cells positively charged relative to medium). This potential may be regarded as a diffusion potential created by the tendency of the diffusible anions to distribute freely between cells and medium but being restrained by the much lower diffusibility of K⁺ (and Na⁺). The potential is not stable because the cell under these abnormal conditions becomes leaky to cations. The escape of K⁺ from the cells promoted by changed membrane permeability, concentration gradient, and potential gradient will diminish the positive membrane potential. This is accompanied by a return of external pH towards initial values as seen in Fig 3.

Measurements of ion distributions by the methods employed in this study are not feasible in a rapidly changing situation as the one described above. However, a

related — albeit less marked and considerably slower — phenomenon may be induced by diluting a sample of heparinized blood with an isotonic solution of non electrolyte. The values given in the lowest line of Table II, show the results of measuring chloride and hydrogen ion distribution in medium and erythrocytes isolated at 38° C, after diluting a 5 ml blood sample with 5 ml of 5 % glucose. As in the case after addition of citrate to the medium fair agreement is found between the distribution of chloride and hydrogen ions, and the values of potential differences calculated from the distribution ratios.

Discussion

The almost parallel course of the regression lines of r_{Cl} and of r_{H} versus plasma pH seen in Fig. 1 suggests a simple relationship between chloride and hydrogen ion distribution in cells and plasma — as would be expected for the distribution of freely diffusible ions in a Donnan system. Contrasting the findings of Peters *et al* (1947) we found that the distribution ratios of Cl^- and H^+ were independent of the way in which pH changes were produced — by altering pCO_2 or by adding acid or base.

A significant difference is found in the absolute values of r_{Cl} and of r_{H} (viz. Table I). At identical plasma pH chloride distribution ratio was found to exceed r_{H} by a mean value of 0.052 (S.D. 0.031, S.E.M. 0.007, $p < 0.001$).

It is not possible to determine the cause of this systematical difference. A difference in the activity coefficients of chloride in cells and plasma, protein binding of chloride trapping of extracellular chloride between the packed cells, and errors arising from liquid junction potentials in the pH measurements of lysed cells are all probable causes.

The Effect of Citrate. Chloride concentration ratio and hydrogen activity ratio were found to be affected to the same degree by the addition of citrate to the external medium (Table II). This supports the conception that both distribution ratios reflect changes of the potential difference between cells and plasma. However it must be admitted that it is not possible to decide whether r_{Cl} or r_{H} gives a fully correct clue to the calculation of membrane potential. Values calculated by the two ratios differ by approximately 2 mV in the pH range considered. Direct recording of the RBC membrane potential has not yet been possible so we have no better estimates than those calculated from r_{Cl} and r_{H} .

The Effects of "Low Electrolyte Media." The term low electrolyte media was employed by Wilbrandt and Schatzmann (1960) to characterize a number of media isotonic to plasma in which RBC exhibits a series of peculiar membrane phenomena due to the fact that electrolytes have been largely or completely replaced by slowly permeating non electrolytes as sucrose, lactose, mannitol or — in the case of human RBC — high concentrations of glucose. For a detailed review of the effects on ion movements, membrane permeabilities and membrane potential the reader is referred to the above cited work. Here shall only be stated that the dysequilibrium caused by the non-electrolyte medium can be appreciated from the pH changes of the medium seen in Fig. 3.

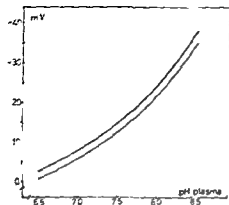


Fig 4

Fig 4 Potential difference between red cells and plasma (calculated by equation 3) as a function of plasma pH. The upper line of the diagram was calculated from r_{Cl} ; the lower line from r_{H} .

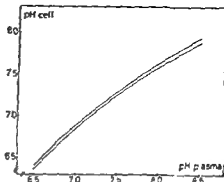


Fig 5

Fig 5 Cellular pH (calculated from equation 4) as a function of plasma pH. The upper line of the diagram was calculated from r_{Cl} ; the lower line from r_{H} .

Peters *et al.* (1947) found that dilution of blood with isotonic sucrose caused the distribution of H⁺ and Cl⁻ to deviate from a Donnan equilibrium. The experiment of Peters *et al.* has been repeated by us (lowest line of Table II). We found that distributions of H⁺ and Cl⁻ were affected in the expected way and to the same degree when blood was diluted with isotonic glucose.

Membrane Potential and Intracellular pH. If the activities of chloride and hydrogen ions could be determined the potential difference (E) between plasma and cells and the hydrogen ion activity of RBC could be calculated. In the following we have attempted to estimate the magnitude of the membrane potential and the intracellular pH of red cells by transposing the Nernst equation inserting the values of r_{Cl} and of r_{H} given by equations 1 and 2. However, it must be born in mind that the ion distribution ratio was calculated from determinations of chloride concentrations. Further pH measurements on lyzed cells may be biased by liquid junction potentials. The following calculations must consequently be taken with reservations.

$$E = 61.7 \log r \text{ mV at } 38^\circ \text{C.} \quad (3)$$

Fig 4 shows the values of E calculated from r_{Cl} and from r_{H} . The membrane potential at a plasma pH of 7.40 is thus found to be a -11 mV as calculated from chloride distribution, and -13 mV when calculated from hydrogen ion distribution.

Intracellular pH was similarly evaluated from the relation

$$\text{pH}_{\text{cell}} = \text{pH plasma} + \log r \quad (4)$$

Fig 5 shows the values of cell pH at various plasma pH levels. The pH of red cells at a plasma pH of 7.40 is thus found to be 7.19 by direct measurement of pH on lyzed cells, and 7.22 when calculated from the chloride distribution.

Water Shifts between Cells and Medium as a Consequence of Donnan Distribution of the Diffusible Anions

The diffusion rates of the anions Cl^- and HCO_3^- through the red cell membrane exceed those of the cations K^+ and Na^+ by a factor of 10^4 . It might therefore be anticipated that the volume changes of red cells caused by acute changes of extracellular pH are due to water movements following the redistribution of diffusible anions.

The calculations below were based on the following assumptions

1) Water is present in osmotic equilibrium on the two sides of the RBC-membrane. Movement of an osmotically active particle from one phase to another causes the concomitant transfer of an equivalent amount of water. At an osmolality of 0.3 osmoles/kg H_2O this equivalent amount is $\frac{1000}{300} = 3.33$ g H_2O per milliosmole.

2) The chloride distribution ratio (eq. 2) is representative of the distribution of all diffusible monovalent anions.

If the concentration of extracellular diffusible anions is kept constant (134 meq $\text{A}^-/\text{kg H}_2\text{O}$), the concentration of A^- in the water phase of the RBC can accordingly be calculated from $r_{(\text{Cl})}$

A^- (pH 7.0) = $134 \times 0.806 = 108.0$ meq/kg H_2O , and

A^- (pH 8.0) = $134 \times 0.447 = 59.9$ meq/kg H_2O

If we consider 1 kg RBC at a plasma pH of 7.0 the following composition may be calculated from the data of Funder and Wieth (1966 a, b)

plasma pH 7.0, RBC 1000 g, cell water 681 g, cell solids 319 g, A^- 108.0 meq/kg RBC H_2O , 73.5 meq/kg RBC. Cell water related to cell solids 2132 g H_2O kg solids

Since, according to assumption 1), one meq A^- moves together with 3.33 g H_2O , the loss of water and of anions that will establish a distribution ratio of 0.447 can be calculated. This is found to be the case in the above example after a loss of 40.8 meq A^- and 134.6 g H_2O respectively. The original one kilogram of RBC considered at pH 7.0 accordingly shrinks by 134.6 g by an increase of extracellular pH from 7.0 to 8.0. We therefore have

plasma pH 8.0 RBC 865 g, cell water 546 g, cell solids 319 g, A^- 59.9 meq/kg RBC H_2O , 32.7 meq/865 g RBC. Cell water related to cell solids 1710 g H_2O kg solids

It is thus found that the amount of water/kg RBC solids is reduced from 2132 g at pH 7.0 to 1710 g at pH 8.0. This calculated reduction of 422 g H_2O kg RBC solids should be compared to the experimental finding of 404 g H_2O kg RBC solids (S.L. 23, n = 15 Funder and Wieth 1966 a). This agreement justifies the conception that the water shift caused by acute change of extracellular pH is almost solely due to the shift of anions secondary to the establishment of a new Donnan equilibrium.

Comparison to Previous Investigations The literature up to 1965 has recently been reviewed in the original study of Bromberg, Theodore Robin and Jensen.

TABLE III Chloride distribution between cells and plasma as a function of plasma pH values from four materials. In the outer right columns are shown chloride distribution ratio at a plasma pH of 7.40 and the values of cellular pH and potential difference between cells and plasma (P D) at plasma pH 7.40 as found by inserting the values of r_{Cl} into eq ns 3 and 4

Author	Relation of r_{Cl} to plasma pH	r_{Cl} at pH _{plasma} 7.40	pH _{cell} calc from r_{Cl}	P D (at 38°C) mV
Dill et al (1937)	$(-0.290 \times \text{pH}_{\text{plasma}}) + 2.820$	0.674	7.229	-10.8
Fitzsimons & Sendroy (1961)	$(-0.344 \times \text{pH}_{\text{plasma}}) + 3.208$	0.662	7.221	-11.1
Bromberg et al (1965)	$(-0.311 \times \text{pH}_{\text{plasma}}) + 2.928$	0.627	7.197	-12.5
Present material	$(-0.359 \times \text{pH}_{\text{plasma}}) + 3.319$	0.662	7.221	-11.0

TABLE IV Hydrogen ion distribution between cells and plasma as a function of plasma pH values from three materials. In the outer right columns are shown hydrogen ion distribution ratio at a plasma pH of 7.40 and the corresponding values of cellular pH and potential difference between cells and plasma (P D) as found by inserting the values of r_{H} into eq ns 3 and 4

Author	Relation of r_{H} to plasma pH	r_{H} at pH _{plasma} 7.40	pH _{cell} calc from r_{H}	P D (at 38°C) mV
Fitzsimons & Sendroy (1961)	$(-0.440 \times \text{pH}_{\text{plasma}}) - 3.883$	0.627	7.197	12.5
Bromberg et al (1965)	$(-0.338 \times \text{pH}_{\text{plasma}}) - 3.062$	0.561	7.149	-15.5
Present material	$(-0.335 \times \text{pH}_{\text{plasma}}) - 3.094$	0.615	7.189	13.0

so it only seems necessary to relate their work to the present, and add a few comments to the older literature on the item.

Table III shows the regression equations of r_{Cl} versus pH of plasma as found by four groups. It is seen that the results agree fairly well.

The hydrogen ion distribution as a function of external pH as found by Bromberg and associates (1965), by Fitzsimons and Sendroy (1961) and by us is shown in Table IV. The slightly lower values of r_{Cl} and of r_{H} as found by Bromberg *et al* (1965) may be explained by the fact that these authors separated cells from plasma at 5–8°C. As the $\Delta \text{pH}/\Delta T$ coefficient is steeper for cells than for plasma, the pH measured on living cells after rewarming to 38° must be expected to be lower, and the pH measured on plasma after separation in the cold and subsequent rewarming correspondingly higher, than the values obtained by separating cells and plasma at the temperature of pH measurement.

Other estimates of intracellular pH in red cells. Attempts to apply the determination of the distribution between cells and plasma of the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) as an indirect measure of H⁺ distribution across the red

cell membrane have not been successful. Thomason (1963) found from studies of DMO distribution that RBC-pH at a plasma pH of 7.35 averaged 7.29, whereas Bromberg *et al.* also employing DMO found pH values, which were markedly lower than the values of pH measured directly on the lysed cells. At a plasma pH of 7.40 the pH of cells was determined to be 7.09 from the DMO distribution, as contrasted by the value 7.15 found by direct pH measurement on lysed cells, and 7.20 as evaluated from r_{Cl} . Bromberg and associates could not offer any explanation of the large discrepancy between the results obtained by Thomason and by themselves by means of DMO. However, these results urge to reluctance in accepting DMO distribution ratios as a true measure of intracellular pH values.

This study was supported by a grant from Fonden til lægevidenskabens fremme. The results were presented at the IX Scandinavian Congress of Clinical Chemistry Sandefjord 1963. The valued technical assistance of Mrs. Karen Meibom is gratefully acknowledged.

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The Action of Insulin on Glycogen Synthesis in Rat Diaphragm

A comparative study of *in vitro* and *in vivo* effects

By

ODDUND SOVIK

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Abstract

SOVIK, O. *The action of insulin on glycogen synthesis in rat diaphragm. A comparative study of in vitro and in vivo effects.* Acta physiol. scand. 1966 68: 246—254.

Insulin administered by intraperitoneal injection in doses which did not significantly alter the

regulation of muscle glycogen synthesis *in vivo*.

In 1960 Villar Palasi and Lerner reported an increased activity of glycogen synthetase (UDP-glucose α -1,4-glucan α -4-glucosyltransferase, EC 2.4.1.11) in rat diaphragm muscle after insulin treatment *in vitro*. Glycogen synthetase has been shown to occur in two forms, one active in the absence of glucose-6-P (independent form, synthetase I), and another which depends upon this ester for its activity (dependent form, synthetase D) (Rosell-Perez *et al.* 1962, Traut and Lipmann 1963).

In the cut diaphragm, originally studied by Villar-Palasi and Lerner, insulin was shown to cause an increased activity of the independent form of the enzyme, without changing the activity measured in the presence of added glucose-6-P. This insulin effect was observed in the absence of glucose or any other substrate in the incubation medium (Villar-Palasi and Lerner 1961) and no concomitant increase in the tissue levels of glucose-6-P could be detected (Lerner *et al.* 1964). It was therefore suggested that insulin stimulated glycogen synthetase D \rightarrow I conversion. This offered an explanation of the so-called "directive" effect of insulin on glycogen synthesis (Beloff-

Chain *et al* 1956, Norman *et al* 1959, Larner *et al* 1960), and suggested an ability of insulin to regulate enzyme activities

The present investigation was undertaken to study the physiological significance of the glycogen synthetase activating effect of insulin. The main question was whether the effect of insulin on the conversion of synthetase D to synthetase I in diaphragm could be reproduced *in vivo*. Rafaelsen (1963) observed that intraperitoneal injection of small quantities of insulin into rats produced a marked stimulation of glycogen synthesis in the diaphragm without any significant effect on blood glucose. With this technique the effect of insulin *in vivo* could be studied under conditions where counter actions from the organism as a whole were minimized.

The results demonstrate that the increased activity of synthetase I in the diaphragm found after insulin *in vitro* was not observed after intraperitoneal injection of insulin. However, the insulin induced glycogen synthesis, determined by the incorporation of ^{14}C -glucose, was greater under *in vivo* conditions.

Methods

In vivo experiments. Rats of the local strain weighing 100–160 g and fasted for 18–24 hrs received an intraperitoneal injection of 1 ml 0.9% NaCl with or without insulin. The injection was made with a morphin cannula to the right of the umbilicus. The standard dose of insulin injected was 10 mU.

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tion and analytical procedures

Analytical techniques. Glycogen synthetase was assayed essentially according to Leloir *et al* (1959). Details of the assay have been described elsewhere. Savik *et al* 1966. The results are expressed as μmoles UDP formed per hour per g wet muscle tissue or per mg muscle protein. The synthetase I

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mon and the supernatant neutralized with 5 M potassium carbonate using methyl orange as an indicator. Glucose 6-P was estimated from the increase in absorbance at $340\text{ m}\mu$ upon addition of 10 μg of 6-P.

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HClO_4 . After centrifugation glucose was determined by the method of Huggett and Nixon (1957).

Materials. Insulin 10 crystallized (specific activity 100 mU/mole) was

TABLE I Acid base parameters in arterial plasma and in brain tissue of rats which were either uninjected or injected with 2 or 3 doses of 100 mg Diamox per kg every second hour. T_{CO_2} is the total CO_2 content of the brain. P_{tCO_2} the mean tissue CO_2 tension. The standard bicarbonate content of the tissue phase was obtained by displacing the experimental points along the buffer curve of the tissue to a CO_2 tension of 40 mm Hg (see Fig. 1).

Rats	Arterial plasma			Brain tissue			
	pH	P_{CO_2} , mm Hg	Actual HCO_3^- , meq/l	T_{CO_2} , mmoles/kg	P_{tCO_2} , mm Hg	Actual HCO_3^- , meq/l	Stand. HCO_3^- , meq/l
Uninjected controls	7.43 ± 0.03	40.2 ± 1.9	24.8 ± 1.1	14.1 ± 0.3	46.5 ± 1.7	16.1 ± 0.3	15.3 ± 0.1
Diamox 100 mg/kg (3 hrs)	7.25 ± 0.02	42.0 ± 1.9	17.3 ± 0.3	15.8 ± 0.3	48.2 ± 1.8	18.6 ± 0.4	17.7 ± 0.4
Diamox 150 mg/kg (6 hrs)	7.22 ± 0.01	46.0 ± 3.1	17.3 ± 1.0	15.6 ± 0.3	51.9 ± 2.9	17.6 ± 0.3	16.2 ± 0.3

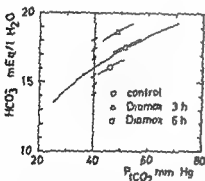


Fig. Relation between the mean bicarbonate content and the tissue CO_2 tension in uninjected rats and in rats injected with 100 mg/kg (3 h) or 150 mg/kg (6 h) of Diamox. The short bars on the 40 mm Hg line denote base excess units.

CL 13,850 had no significant effect on the tissue bicarbonate content. Control experiments further showed that there was no increase in the CSF bicarbonate concentration after acetazolamide at these equilibration times. This indicates that the observed increase in the tissue bicarbonate represented an increase in the intracellular bicarbonate concentration. The mechanisms behind this increase may be related to the action of the carbonic anhydrase in the tissue cells. These mechanisms are being further investigated.

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Chambers *et al.* 1956 Norman *et al.* 1959 Larner *et al.* 1960) and established an effect of insulin to regulate enzyme activities

The present investigation was undertaken to study the physiological effect of the glycogen synthetase activating effect of insulin. The main objectives were: (1) the effect of insulin on the conversion of synthetase D to synthetase I could be reproduced *in vivo*; (2) Rafsaelsen (1963) observed that intraperitoneal injection of small quantities of insulin into rats produced a marked stimulation of glycogen synthesis in the diaphragm without any significant effect on blood glucose; (3) by this technique the effect of insulin *in vivo* could be studied under conditions in which counter actions from the organism as a whole were minimized.

The results demonstrate that the increased activity of synthetase I in the diaphragm found after insulin *in vitro* was not observed after intraperitoneal injection of insulin. However, the insulin induced glycogen synthesis determined by incorporation of ^{14}C glucose was greater under *in vivo* conditions.

Methods

Insulin. Insulin was prepared from the crystalline zinc salt of insulin (Novo) by the method of Huggins and Dixon (1957). Protein was determined as described by Lowry *et al.* (1951). Insulin (10 units) was obtained from Novo Industri A/S (Copenhagen, Denmark). UDP-glucose (specific activity $> 100 \text{ mCi/mmole}$) was purchased from New England Nuclear Corporation. UDP-glucose samples drawn from the tail vessels were deproteinized with 0.33 M HClO₄. After centrifugation glucose was determined in the supernatant by the glucose oxidase method of Huggins and Dixon (1957). Protein was determined as described by Lowry *et al.* (1951). Insulin (10 units) was obtained from Novo Industri A/S (Copenhagen, Denmark). UDP-glucose (specific activity $> 100 \text{ mCi/mmole}$) was purchased from New England Nuclear Corporation. UDP-glucose samples drawn from the tail vessels were deproteinized with 0.33 M HClO₄. After centrifugation glucose was determined in the supernatant by the glucose oxidase method of Huggins and Dixon (1957). Protein was determined as described by Lowry *et al.* (1951). Insulin (10 units) was obtained from Novo Industri A/S (Copenhagen, Denmark). UDP-glucose (specific activity $> 100 \text{ mCi/mmole}$) was purchased from New England Nuclear Corporation.

TABLE 1 Effect of insulin on glycogen synthesis in the isolated intact diaphragm and in diaphragm *in situ*. The isolated diaphragms were incubated as described under * Methods. In the *in vivo* experiments the animals received intraperitoneal injections of 2 μ C¹⁴-glucose with or without insulin. In some experiments samples for determination of blood glucose were taken 60–90 min after the injection. The animals were killed after 150 min. Blood glucose, amount of glycogen and labelling of glycogen by ¹⁴C-glucose were determined as described under * Analytical techniques. The number of observations is given in parentheses.

Experimental conditions	Glycogen		Blood glucose
	μ g/100 mg wet tissue ¹	c.p.m./100 mg wet tissue ²	mg/100 ml
<i>Diaphragm in situ</i>			
Control	264 \pm 32 (6)	285 \pm 103 (6)	114.3 \pm 4.1 (5)
Insulin	1188 \pm 90 (6)	11838 \pm 2530 (6)	109.5 \pm 2.6 (6)
<i>Isolated diaphragm</i>			
Control	166 \pm 19 (9)	1417 \pm 228 (9)	
Insulin	165 \pm 18 (9)	8533 \pm 1320 (9)	

¹ \pm Standard error of the mean.

² Glucose, glycogen, glucose-6-P, phosphoenolpyruvate, pyruvate kinase and NADP were purchased from Sigma Chemical Corporation. Glycogen was passed over a column of Norit before use. Glucose-6-P dehydrogenase was obtained from C.F. Boehringer & Soehne. Chemical and biochemical reagents not mentioned above were of standard analytical grade.

Statistical methods: Each observation is based upon analysis in duplicate of muscle tissue or blood from one animal. The significance of a difference between two groups of animals was calculated by Student's *t* test. *P* values were obtained from Coxton (1959).

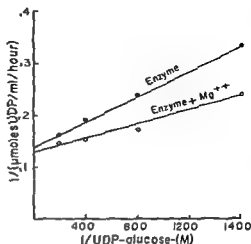
Results

Effect of insulin on glycogen synthesis. Table 1 shows the effect of insulin on glycogen synthesis in the isolated diaphragm compared with the diaphragm *in situ*. *In vitro* the labelling of glycogen by ¹⁴C-glucose was increased 6-fold by insulin 0.1 U/ml. This insulin effect was not essentially increased by changing the incubation time by adding substrate to the medium, or by increasing the hormone dose. Intraperitoneal injection of insulin 10 mL/100 g had a more marked effect on glycogen synthesis. Measured 150 min after insulin injection the amount of glycogen in diaphragms from insulin treated animals was 4.5 times higher than in the controls. The amount of glycogen in the isolated diaphragm from insulin treated animals was also increased 4.5 times. As demonstrated in Figure 1, the isolated diaphragm levelled off 120 min after insulin injection. The blood glucose of insulin treated animals tested 60–90 min after the injection, did not differ significantly from controls (Table 1).

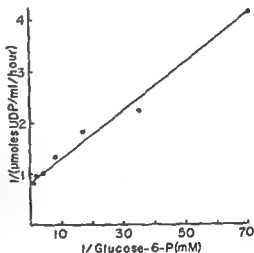
Characterization of glycogen synthetase. When a crude enzyme extract containing about 90% synthetase I was studied, the apparent K_m for UDP-glucose was 9×10^{-4} M (Fig. 1). This is similar to values previously reported for the rat muscle enzyme (Rosell Perez *et al.* 1962). Mg²⁺ lowered the K_m -value without affecting V_{max} .

EDTA pH 7.8 The homogenate was centri-

to the standard procedure, except for varying the concentration of UDP glucose. In some experiments Mg^{++} was added



for 20 min and the supernatant assayed for glycogen synthetase according to the standard procedure, except for varying the concentration of glucose 6-P



The effect of varying the concentration of glucose 6-P was also studied (Fig 2). The apparent K_A for the activation of synthetase D by glucose-6-P was $5.5 \times 10^{-4} M$, a value close to that reported by Leloir *et al.* (1959).

Effect of insulin on glycogen synthetase The activity of glycogen synthetase was studied in extracts from diaphragms incubated with insulin (Table II). Insulin caused a rise in synthetase I from 9.4 to 17.9%, while the total enzyme activity remained unchanged. Table II also shows activities of glycogen synthetase in extracts from diaphragms after insulin *in vivo*. In contrast to the results obtained with diaphragm *in vitro*, insulin had no effect on the amount of synthetase I. The percentage of synthetase I was almost unchanged throughout 4 hrs after insulin injection (I). The same result was obtained when UDP-¹⁴C-glucose was used for gly

TABLE II Effect of insulin on glycogen synthetase in the isolated intact diaphragm and in diaphragm *in situ*. The experimental conditions were similar to those described in the legend of Table I except that ^{14}C -glucose was omitted. Glycogen synthetase was assayed as described under Analytical techniques. The number of observations is given in parentheses.

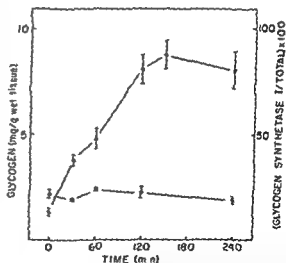
Experimental conditions	Glycogen synthetase		Percentage of I form
	No glucose 6 P	Glucose 6 P added	
<i>µmoles UDP/g wet tissue/hr¹</i>			
<i>Isolated diaphragm</i>			
Control	131 ± 24 (9)	140.0 ± 7.4 (9)	9.4
Insulin	25.2 ± 3.4 (9)	142.0 ± 6.4 (9)	17.9
<i>Diaphragm in situ</i>			
Control	18.3 ± 2.1 (6)	202.0 ± 14.3 (6)	9.0
Insulin	15.6 ± 1.0 (6)	177.5 ± 15.3 (6)	8.7
<i>µmoles UDP/mg protein/hr¹</i>			
Control	0.99 ± 0.29 (4)	8.78 ± 0.21 (4)	
Insulin	0.97 ± 0.43 (4)	8.52 ± 0.56 (4)	

¹ ± Standard error of the mean

² p = 0.01

Fig. 3 Percentage of synthetase I and amount of glycogen in diaphragms after intraperitoneal injection of insulin. Insulin was administered according to the standard procedure and the animals were killed after the time periods indicated. Glycogen and glycogen synthetase were assayed as described under analytical techniques. One hemidiaphragm was used for glycogen while the other was used for enzyme determination. Each value for glycogen represents the mean of 5-10 and each value for glycogen synthetase the mean of > 6 observations. The length of the vertical bars shows two standard errors of the mean.

Glycogen ●—●
synthetase I ○—○



thetase determination. In some of the *in vivo* experiments insulin seemed to suppress the enzyme activities. Table II. This effect was not present when the muscle protein, rather than the wet weight of the muscle, was used as a reference for the enzyme activity. The reason for the effect is supposedly an increased tissue weight after insulin injection due to glycogen accumulation.

TABLE III Amount of glycogen and synthetase I in the $105\,000 \times g$ particulate fraction of diaphragm muscle homogenate after insulin *in vivo*. In each experiment insulin was administered to 3 rats according to the standard procedure while 5 rats received saline. The animals were killed after 150 min and hemidiaphragms were cut out and immediately frozen in liquid O_2 . The following procedure was carried out in the cold room ($2^\circ C$). The experimental as well as the control diaphragms were pooled, powdered and extracted (1:20 w/v) with $0.05\,M$ Tris— $0.005\,M$ EDTA pH 7.8 in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at $5\,000 \times g$ for 10 min. The sediment was discarded while 12 ml of the supernatant was centrifuged in a Spinco ultracentrifuge (Model L) for 3 hrs. The small sediment was homogenized with 3 ml of the original buffer and analyzed for glycogen and enzyme as described under "Analytical techniques". The number of observations is given in parentheses.

Experimental conditions	Glycogen	Glycogen synthetase
	$\mu g/ml^1$	percentage of I form ²
Control	25.7 ± 12.8 (5)	18.7 ± 1.5 (3)
Insulin	214.4 ± 50.9 (3)	18.4 ± 0.7 (3)

¹ \pm Standard error of the mean

TABLE IV Effect of insulin on glucose 6-P in the diaphragm. The experimental conditions were as described in the legend of Table I, except for the omission of ^{14}C -glucose. Glucose-6-P was determined as described under "Analytical techniques". The number of observations is given in parentheses.

Experimental conditions	Glucose 6-P
	$\mu moles/g$ wet tissue
Diaphragm <i>in situ</i>	
Control	0.76 ± 0.06 (9)
Insulin	1.15 ± 0.11 (9)
Isolated diaphragm	
Control	0.10 ± 0.04 (4)
Insulin	0.09 ± 0.04 (4)

¹ \pm Standard error of the mean

² $p < 0.01$

The activity of glycogen synthetase in the $105\,000 \times g$ particulate fraction of the diaphragm muscle homogenate was studied (Table III). In this fraction the specific activity of the enzyme was 18–19 fold higher than in the crude muscle extract. While insulin *in vivo* caused a 10-fold increase in the amount of glycogen in this fraction, the percentage of synthetase I remained unchanged.

The possibility was tested that insulin injection and subsequent incubation of the diaphragm might reveal a hormone effect on synthetase I. Rats were

min after intraperitoneal injection of insulin. Intact diaphragms were prepared and incubated 60 min at 37° C in Krebs Ringer phosphate buffer with glucose (2 mg/ml). However, the amount of synthetase I was the same in diaphragms from insulin-treated and control animals.

Effect of insulin on glucose 6 P. In diaphragms *in vitro* the glucose 6 P levels were low, and there was no effect of adding insulin to the incubation medium (Table IV). The basic levels of glucose 6-P determined in diaphragms frozen immediately after excision were considerably higher, and close to the apparent K_m for synthetase D (Fig. 2). The amount of glucose 6-P was compared in diaphragms removed 150 min after intraperitoneal injection of saline with and without insulin. After insulin the amount of glucose 6 P was substantially increased, and reached values well above the apparent K_m for synthetase D.

Discussion

The increase in glycogen synthetase I in rat diaphragm after incubation with insulin is well documented (Villar Palasi and Larner 1960, Danforth 1965), and is also demonstrated in the present work. The glycogen synthetase D \rightleftharpoons I interconversion probably involves phosphorylation by a specific kinase and dephosphorylation by a specific phosphatase (Friedman and Larner 1963). Whether insulin inhibits the kinase or stimulates the phosphatase system is unknown.

In contrast to the *in vitro* results, intraperitoneal injection of insulin did not cause any increase of synthetase I in the diaphragm. There are two possible explanations for the striking discrepancy between the *in vivo* and the *in vitro* results. Firstly, the increase of synthetase I might be reversed during preparation and handling of the tissue. Secondly, the effect of insulin on synthetase I thought present *in vitro*, might not occur in the intact rat organism. The removal of an organ or a tissue frequently causes adrenalin secretion, muscle convulsions or anoxia. Adrenalin will suppress the total glycogen synthetase activity (Belocoprnow 1961), as well as the amount of the I form (Larner *et al* 1963, Danforth 1965). Muscle convulsions (Danforth 1965) or anoxia (Sovik *et al* 1966) will increase synthetase I activity. For the following reasons these factors were supposed to be of minor importance: 1. The percentage of synthetase I was found to be the same in diaphragms prepared after decapitation of the animals and when prepared under anesthesia. 2. A similar percentage of synthetase I was observed in diaphragms incubated under basal aerobic conditions. 3. The synthetase D \rightleftharpoons I interconversion in muscle is supposed to operate in minutes rather than seconds (Danforth 1965). In the present investigation the diaphragm was always frozen within one minute after the animals were killed.

The diaphragm *in vivo* is performing rhythmical mechanical work whereas the isolated diaphragm is a resting muscle, unless stimulated by the isolated *nervus phrenicus*. However, information on the effect of mechanical work on glycogen synthetase activity is lacking, and it is at present difficult to explain the observed discrepancy between the *in vivo* and the *in vitro* results in terms of difference in mechanical work.

The effect of intraperitoneal injected insulin on glycogen deposition in the diaphragm is dramatic. It occurs with doses of insulin too small to alter the concentration of blood glucose. The observation that synthetase I remains unchanged suggests that insulin *in vivo* can markedly enhance glycogen synthesis in this muscle without causing any synthetase D \rightarrow I conversion.

It is debatable whether, in the absence of an insulin effect on synthetase I, the effect of insulin on glucose transport (Park *et al* 1955, Park and Johnson 1955) could account for the increased glycogenesis. It has been claimed (Norman *et al* 1959) that when glucose entry into diaphragm is increased by raising the glucose concentration in the incubation medium, no stimulation of glycogen synthesis comparable to that induced by insulin occur. This conclusion was based upon experiments *in vitro*, and whether it is valid under *in vivo* conditions is not known.

Glucose-6 P might play an important role in the regulation of muscle glycogenesis *in vivo*. After intraperitoneal injection of insulin the concentration of glucose 6 P in diaphragm was raised by 50 per cent, and reached a value above the apparent K_m for synthetase D. Although formation of glucose 6 P from glycogen during preparation of the tissue might have contributed somewhat to the glucose 6 P levels observed, this would not change the absolute difference observed with and without insulin. It is likely that the change in glucose 6 P levels in response to insulin caused a substantial increase in the total glycogen synthetase activity.

The author would like to thank Professor O. Walaas for his continuous interest in the present investigation. This work was supported by grants from Nordic Insulin Fund and from The Norwegian Council for Science and The Humanities. The technical assistance of Mrs. A. Adler and Mrs. A. Eggevik is acknowledged.

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TABLE I Acid base parameters in arterial plasma and in brain tissue of rats which were either uninjected or injected with 2 or 3 doses of 50 mg Diamox per kg every second hour. T_{CO_2} is the total CO_2 content of the brain. P_tCO_2 the mean tissue CO_2 tension. The standard bicarbonate content of the tissue phase was obtained by displacing the experimental points along the buffer curve of the tissue to a CO_2 tension of 40 mm Hg (see Fig.)

Rats	Arterial plasma			Brain tissue			
	pH	P_{CO_2} , mmHg	Actual HCO_3^- , meq/l	T_{CO_2} , mmoles/kg	P_tCO_2 , mmHg	Actual HCO_3^- , meq/l	Stand HCO_3^- , meq/l
Uninjected controls	7.43 ± 0.01	40.2 ± 1.9	24.8 ± 1.1	14.1 ± 0.3	46.1 ± 1.7	16.1 ± 0.3	15.3 ± 0.1
Diamox 100 mg/kg (3 hrs)	7.26 ± 0.02	42.0 ± 1.9	17.3 ± 0.3	15.8 ± 0.3	49.2 ± 1.8	18.6 ± 0.4	17.7 ± 0.4
Diamox 150 mg/kg (6 hrs)	7.22 ± 0.01	46.0 ± 3.1	17.3 ± 1.0	15.6 ± 0.3	51.9 ± 2.9	17.6 ± 0.3	16.2 ± 0.3

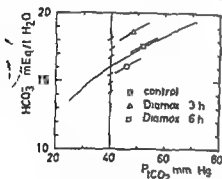


Fig. Relation between the mean bicarbonate content and the tissue CO_2 tension in uninjected rats and in rats injected with 100 mg/kg (3 h) or 150 mg/kg (6 h) of Diamox. The short bars on the 40 mm Hg line denote base excess units.

CL 13,800 had no significant effect on the tissue bicarbonate content. Control experiments further showed that there was no increase in the CSF bicarbonate concentration after acetazolamide at these equilibration times. This indicates that the observed increase in the tissue bicarbonate represented an increase in the intracellular bicarbonate concentration. The mechanisms behind this increase may be related to the action of the carbonic anhydrase in the tissue cells. These mechanisms are being further investigated.

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Inhibitory Action of Adrenergic Blocking Agents on Catecholamine Release and Uptake in Isolated Nerve Granules

By

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Abstract

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A series of adrenergic α and β blockers have been found to decrease the release rate of noradrenaline from a suspension of bovine splenic nerve granules incubated in phosphate buffer pH 7.5. The same blockers also inhibited the uptake of noradrenaline in partially depleted nerve granules in the presence of ATP-Mg. It is tentatively suggested that the interactions of the adrenergic blocking agents with uptake mechanisms for noradrenaline in the nerve granules and at the receptor level in effector cells involve certain common features.

In the course of a study of various autonomic drugs on the release rate of noradrenaline (NA) from isolated bovine splenic nerve granules incubated in isotonic potassium phosphate at pH 7.5, it was observed that phenoxibenzamine (PBA) caused a marked inhibition of the spontaneous NA release and also inhibited the ATP-dependent uptake of NA in partially depleted nerve granules. Even stronger effects were noticed with dichloroisoproterenol (DCI) and chlorpromazine (Euler and Lishajko 1961, 1965). In the present paper we wish to report the action of a number of adrenergic blocking agents, both of the α and β receptor blocking series on the release rate of NA and on the ATP dependent amine uptake.

Methods

Nerve granules were obtained from bovine splenic nerves either by squeezing the desheathed nerves between nylon rollers or by homogenization with an Ultra Turrax apparatus (Janke & Kunkel, Staufen, Br.). After removal of the loose tissue material and larger particles by centrifugation 10 min at 9 000 \times g the granule suspension in the supernatant was incubated at 20 °C in 0.13 M potassium phosphate buffer at pH 7.5 for 30—90 min with and without the blocking drugs in concentrations as stated in the experimental part.

¹ Abbreviations used: NA, noradrenaline; A, adrenaline; PBA, (phenoxibenzamine); DCI, (dichloroisoproterenol).

TABLE 1 Relative release rate (control = 1) for NA in bovine splenic nerve granule suspension incubated for 60 min at 20 °C in phosphate buffer, pH 7.5 Mean of 2-6 expts

	Molar concentration	
	2×10^{-5}	3×10^{-5}
<i>α-receptor blocker</i>		
SY 28		0.30 (0.25-0.35)
Phenoxybenzamine		0.32 (0.24-0.43)
Azapetine (Ildar)		0.33 (0.29-0.39)
Phentolamine		0.40 (0.14-0.84)
Hydergin	0.30 (0.20-0.35)	
Chlorpromazine	0.47 (1 expt.)	
<i>β-receptor blocker</i>		
Propranolol		0.38 (0.15-0.58)
H 56/28		0.42 (0.33-0.61)
DCI		0.42 (0.30-0.56)
Pronethalol		0.49 (0.42-0.54)
Butoxamine		0.54 (0.36-0.79)
N isopropyl methoxamine		0.56 (0.35-0.79)
NJ 1998		0.86 (0.77-0.94)
NJ 1999		1.2 (1.1-1.2)

In the uptake experiments the nerve granules were first partially depleted by incubation for 10 min at 37 °C in phosphate buffer at pH 7.5 and then further incubated for 30 min at 20 °C in the presence of NA (6×10^{-5} M), ATP (3 mM) and Mg²⁺ (3 mM) with and without the drugs.

Since the NA release follows an approximately exponential course the relative release rate of NA in the presence of the drugs may be expressed as the ratio

$$\frac{\log NA_0 - \log NA_D}{\log NA_0 - \log NA_C}$$

where NA_0 is the original amount of NA, NA_D is the remaining NA in the presence of the drug and NA_C the remaining NA in the control after incubation. The action of the drugs on the uptake of NA is expressed as the ratio of the NA uptakes with and without the drug. Uptake is taken as the net difference in NA values between the sediments before and after the second incubation for 30 min at 20 °C.

The following drugs were used:

Phenoxybenzamine (Dibenzylinc[®] Smith Kline & French Philadelphia), phentolamine (Regitiner[®] CIBA, Basel), Hydergin[®] (Sandoz, Basel), SY-28 (N- α -naphthylmethyl-N-ethyl- β -bromoethylamine (Parke, Davis & Co., Ann Arbor), azapetine phosphate (Ildar, Hoffmann-La

gus of the drugs.

Results

Effects on the NA release rate

The α blockers tested, PBA, phentolamine, Hydergin, azapetine, SY 28 and chlorpromazine, all lowered the NA release rate from isolated splenic nerve granules as seen in Table 1. On a molar concentration basis Hydergin had the strongest effect while chlorpromazine was least active in this respect.

TABLE II Relative uptake of NA in partially depleted bovine splenic nerve in the presence of 3 mM ATP and 3 mM MgCl₂ and various adrenergic blockers. Mean and range (control uptake = 1)

	Molar concentration 2×10^{-5}	3×10^{-4}
<i>α receptor blocker</i>		
Azapetine		0.10
Phentolamine		0.38 (0.37-0.38)
56/28		0.61
Phenoxybenzamine		0.62 (0.57-0.68)
Chlorpromazine	0.46	
Hydergin	0.60 (0.53-0.66)	
<i>β-receptor blocker</i>		
Pronethalol		0.19 (0.07-0.25)
DCI		0.22 (0.08-0.37)
Propranolol		0.25 (0.22-0.28)
56/28		0.31 (0.30-0.31)
Butoxamine		0.45
MJ 1999		0.73
N-isopropyl methoxamine		0.74 (0.66-0.81)
MJ 1998		0.93

Of the β blockers tested (Table I) all had an inhibitory effect on the release rate except MJ 1999, while MJ 1998 had a weak action only. The strongest actions were seen with propranolol, 56/28, DCI and pronethalol at a concentration of 3×10^{-4} M.

Effect of adrenergic α and β blockers on the NA uptake in partially depleted granules in the presence of ATP Mg

The results obtained with various adrenergic blocking substances on the release of NA from isolated nerve granules made it of interest to study also their effect on the amine uptake. As shown previously (Euler and Lishajko 1963) addition of ATP greatly enhances the amine uptake in partially depleted nerve granules and we have studied the effects of the drugs on this stimulated uptake.

As seen in Table II all of the blockers inhibited the uptake of NA in the presence of ATP Mg. All drugs except Hydergin and chlorpromazine were tested in a concentration of 3×10^{-4} M which caused a maximal or near maximal inhibition of the spontaneous release. Hydergin was not tested in a higher concentration than 2×10^{-5} M since this concentration was highly effective on the inhibition of spontaneous release.

As seen in Table II the strongest inhibitory effect on the NA uptake was exerted by the α blocker azapetine and by the β blocker pronethalol. The β blockers N-isopropylmethoxamine MJ 1998 and MJ 1999 had only a weak effect and Hydergin

On preincubation of the granule suspension for 30 min at 2° C with the drugs, the inhibitory effect on the uptake of NA was greater. The most marked effects were then observed with DCI, propranolol and pronethalol.

Discussion

A wide variety of drugs with different kinds of action have been found to influence the amount of tritiated NA in various organs after injection (Axelrod, Whitby and Hertting 1961). By administration of the drug before or after the radioactive NA certain conclusions could be drawn as to whether the drug caused a release of NA or inhibited the uptake (Axelrod, Hertting and Potter 1962). From these studies it emerged that among several drugs, chlorpromazine and DCI blocked the entry of NA without causing a release.

From the present, extended study it can be seen that inhibition of NA release from splenic nerve granules as well as inhibition of the ATP-dependent uptake is a common property of almost all of the adrenergic receptor blocking substances studied. The term "ATP-dependent uptake" represents approximately the NA uptake in the presence of ATP-Mg, since the NA uptake without addition of ATP-Mg is small under the prevailing conditions.

No strict parallelism was observed between the effect of the drugs on the release rate and that on the NA uptake. Thus Hydargin had a more marked effect on the release rate than on the uptake, and the same was noted for *N*-isopropyl methoxamine. The latter compound has been reported to be less active than, for instance, pronethalol (Hardman, Barboriak and Meester 1963) in its β blocking action *in vivo*. MJ 1998 and 1999 have been the only adrenergic receptor blockers so far studied which exert only a negligible action on the granules. Whether this is to some extent associated with their reportedly low lipid solubility is still unclear (Kvam, Riggilo and Lish 1965). No definite relationship between the pharmacological or clinical effects of the various blockers and their inhibitory action of the NA release rate and uptake in splenic nerve granules has been shown to occur.

The two chemically closely related compounds pronethalol and propranolol both have very marked inhibitory effects on the NA release rate as well as on the NA uptake in the presence of ATP-Mg. With regard to their actions *in vivo* propranolol is reported to have some 5–10 times stronger action than pronethalol.

If in fact there is more than a fortuitous connection between the adrenergic blocking function and the effect on the NA release rate or on the ATP-dependent amine uptake in isolated granules, it is tempting to relate it to a common effect on a transmitter uptake in which possibly ATP is involved. The importance of ATP for the uptake of NA has been demonstrated in different ways. Addition of ATP to the incubation medium diminishes or prevents the spontaneous net loss of NA from nerve granules, owing largely to a compensatory uptake of NA when this amine is present. Furthermore, after partial depletion of NA from the granules following incubation, ATP greatly enhances the uptake of exogenous NA. Euler and La-

hajko 1963), as observed also for adrenal medullary granules (Carlsson Hillarp and Waldeck 1963)

The protective effect of NA and ATP by which the NA content of the granules is maintained during incubation, is to some extent counteracted by PBA, which although it strongly lowers the spontaneous release rate to about 1/8 of the control rate in a concentration of 7.4×10^{-4} M exerts a smaller protective effect at this concentration than at 3×10^{-4} M. This may be explained by assuming that the protective effect is dependent on an uptake which is inhibited by PBA. A still stronger inhibition of the "protective" effect of ATP was observed in the present study with propranolol 3×10^{-4} M which removed about 80 per cent of the ATP effect.

As to the spontaneous NA release from granules Euler Lishajko and Syärne (1963) have reported that the amine release is relatively faster than the accompanying ATP release which causes a conspicuous fall in the amine/ATP ratio as incubation proceeds. Preliminary experiments seem to indicate that the drugs under study alter this ratio (Euler and Syärne unpublished obs.).

An interaction of adrenergic agents with uptake of NA in organs has been previously observed (Axelrod *et al.* 1961, 1962; Rosell and Axelrod 1963) and it has been assumed that such agents prevent the binding and subsequent destruction of the released transmitter at the receptor level (Brown and Gillespie 1959; Schapiro 1958). The increased overflow of transmitter from the spleen has also been interpreted as inhibition of reuptake (Blakeley, Brown and Geffen 1964; Karpekar and Cervoni 1963) have concluded from their experiments with various adrenergic blocking agents that the tissue receptors are involved in a major way in inactivating neurally released transmitter. On the other hand Burgen and Iversen (1963) find no support for the view that the uptake of catecholamines is explicable in terms of a binding at sympathetic α or β receptors.

The present observations appear to give the first indication of a common antagonistic effect of a specific group of pharmacologically active substances on the isolated granule system and on effector cells. The results are thus suggestive of an NA uptake mechanism bound to receptors both in granules and in effector cells which can be blocked by the same kind of substances. Whether or not the uptake of NA in the receptor of the effector cell is ATP dependent is not experimentally proven but it has been postulated (Belleau 1960) that ATP might have an important function in this system.

Should a functional similarity in the NA uptake mechanism between nerve granules and the adjacent synaptic receptor pertain to this might conceivably reflect some association between the effector cell and the outgrowing adrenergic axons which make contacts at an early stage during development.

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By

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Retained 12 April 1966

Abstract

An attempt has been made to define the types of muscle fibres present in the iliofibularis muscle of *Tropus* (see ■). Histochemical studies were carried out on cross sections of the muscle. The variation between cells in the content of lipids, DP, diaphorase and succinic dehydrogenase was noted, and on this basis the fibres were classified into three main groups. Single living fibres representing the histochemical groups were selected and their contractile behaviour in response to electrical stimuli on and the application of ACh was studied. The findings indicated a relationship between the histochemical and functional properties of the muscle fibres. Fibres with a moderate content of DP and enzymes (pale fibres) were fast fibres which fatigued rapidly on "

Fibres with a high content of lipid rapidly. Fibres containing no lipid were also fibres. Staining of the of varying gross morphology and grapple end plates with very low

44 67

Amphibian skeletal muscle is generally regarded as being composed of two types of muscle fibres: fast and slow (Tasaki and Mizutani 1944; Kuffler and Gerard 1947). A stimulus which depolarizes a fast fibre to or beyond a threshold causes a propagated electrical impulse and a twitch. Slow fibres however show no regenerative electrical activity and respond to a local depolarization with a local contraction (see review by Peachey 1961). Structural differences between fast and slow muscle fibres have also been described (Page 196). The evidence which indicates the presence of these two groups of muscle fibres does not preclude the existence of sub-types within either group of fibres, nor even of the existence of entirely unrecognized groups of muscle fibres. Shamanna (1962, 1963) description of fibres with properties intermediate between those of fast and slow fibres can be taken to be a case in point, although the validity of these results has been doubted (Orkand 1963).

The object of the present investigation was to determine the function muscle fibres present in a toad skeletal muscle. First, the histochemi-

of all the fibres in the muscle were determined from cross sections of the muscle, and the fibres were separated into histochemical groups. Fibres representing the histochemical groups were isolated and their contractile behaviour was investigated. It will be shown that in *Xenopus laevis* the contractile properties of individual fibres can be related to the histochemical properties of the fibres.

Fast and slow fibres have distinctly different histochemical properties, and the fast group of fibres may be subdivided on both histochemical and functional grounds. This is consistent with an earlier finding in the frog *R. ridibunda* (Lännergren 1965).

Methods

The experiments were performed if not stated otherwise on the iliofibularis muscle of the toad *Xenopus laevis*. Both male and female toads were used of weights ranging between 40 and 80 g. The animals were kept at room temperature and were fed minced meat once a week. Histological investigations were performed on muscles excised between January and September while the observations of the contractile behaviour of the muscle fibres were made during July–September.

Histological procedures

Cross sections of muscle were stained for lipids, succinic dehydrogenase and diphosphopyridine nucleotide-diaphorase (DPN-diaphorase). The procedures used were:

(a) Lipids were stained with Sudan Black B (Carleton and Drury 1957). Staining was carried out partly on 5 μ m sections of tissue which had been fixed in formalin and embedded in Carbowax and partly on 10–20 μ m sections of frozen fresh tissue.

(b) Succinic dehydrogenase was demonstrated by the method of Nachlas *et al.* (1957).

(c) DPN-diaphorase was demonstrated using the method of Scarpelli, Hess and Pearse as described by Pearse (1960). Nitro-BT was used as the electron acceptor.

Prior to the procedures (b) and (c) the animals were cooled to about 0°C, then pitied and an iliofibularis muscle was excised. The muscle was then immediately frozen in liquid propane at -190°C and subsequently stored at -65°C for periods not longer than two weeks. Serial 10–20 μ m sections were cut on a cryostat and alternate sections were used for demonstrating enzymes or lipids.

Cholinesterase at the end plates of single muscle fibres or on small bundles of fibres was demonstrated by the thiolacetic acid method as published by Barnett and Palade (1958).

Nerve terminals were stained using a Kongal et al. ethylene blue method (Romeis 1958) on isolated muscle fibres.

Single fibre dissection

The excised iliofibularis muscle was held by its tendons between split nylon rods in a shallow Perspex dish containing Ringer's solution (Fig. 1). Single muscle fibres were then isolated with the aid of finely ground forceps and scissors.

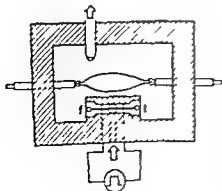


Fig. 1. Schematic drawing of the cell used for dissection and recording. The muscle was held between split nylon rods during the dissection of a single fibre. After dissection the fibre was attached to the glass rods and moved in the Ringer's solution to a gutter where a cover-slip was placed over it. The rod was fixed directly to a micro-manipulator; the rod was attached to the movable plate of a transducer which was mounted on a second micro-manipulator. Flow of Ringer's solution and caterpillar was

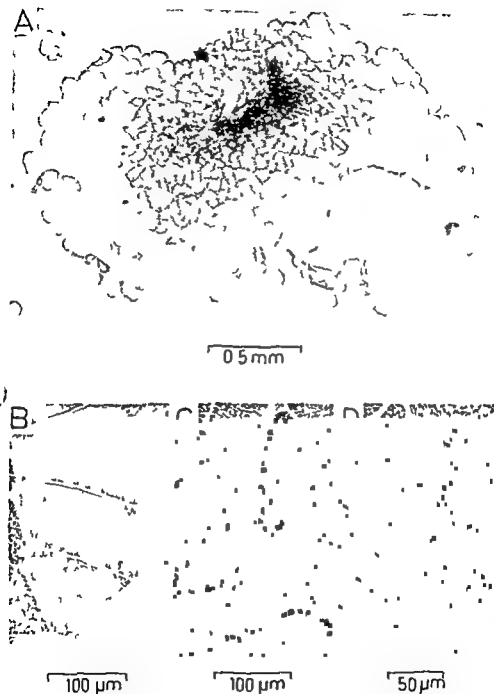


PLATE 1

A. Cross-section of a whole tibularis muscle fixed in formalin, embedded in Carbowax 4.

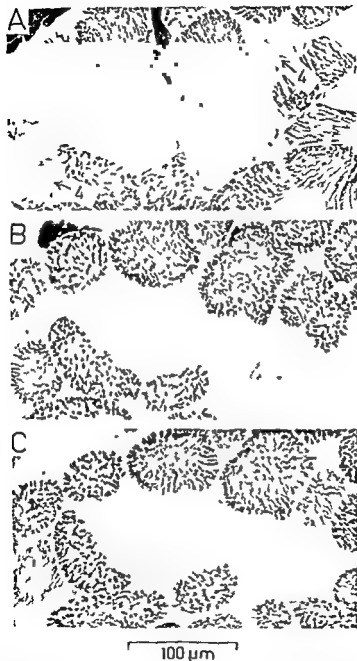


PLATE 2

Frozen 20 μm serial sections from centre of dark zone of fibular muscle. *A* Section stained with Sudan Black B. *B* DPN-diphosphorase staining. *C* Succinate dehydrogenase staining. Alternate sections are stained allowing the same fibre to be followed through the sections. Four non-stained fibres can be seen in the centre of all sections and can be used for orientation. Two fibres of the type in column 4 of Table 1 are indicated by arrows and number 4.

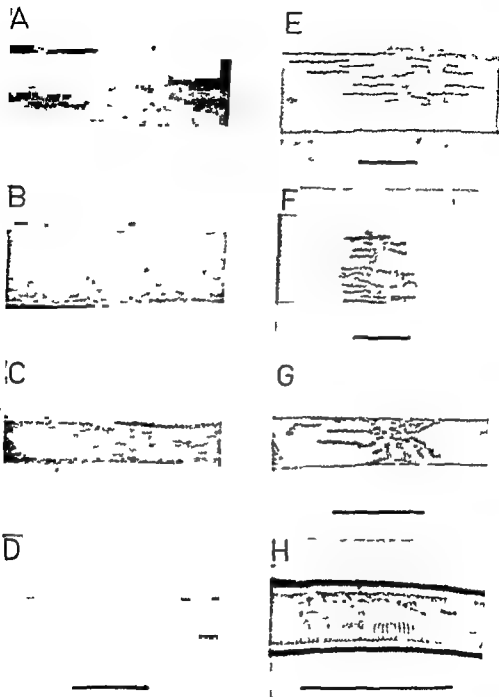


PLATE 3

A, B, C, and D. Appearance of single living muscle fibres in dark field illumination. They are of the types described in Table 1, column 1, 2, 3 and 4 respectively. *E, F, and G.* Corresponding fibres stained by the thioflavin acid method. *H.* Nerve ending on fibre stained on a clear fibre (Table 1, column 5). Methylene blue. Oil immersion. *A-H.* Fibres *E, F, and G* flattened between slide and coverslip. Scale is the same for *A-H*. Scale bars 100 μ .

TABLE 1 Properties of the different muscle fibres in the iliofibularis muscle

s. laevis

	1	2	3	4	5
Location (p = peripheral c = central)	p	c	c	c	
Approx. no. of fibres	350	200	50	50	
Diameter in μm	100-150	55-110	40-70	3	
Affinity to stain					
Sudan Black B	+	++	—	—	
DPN-diaphorase specific method	+	+++	++	—	
SDH-specific method	+	+++	++	—	
Nomenclature	pale	dark	dark	—	

zone of the iliofibularis muscle which, to some extent, deviated from the scheme. The properties of the deviant fibres are given in columns 3 and 4 of Table 1. In Plate 2 two fibres of the type in column 4 are indicated by arrows and number 4.

Selection of single living fibres representing the histochemical groups

Individual living fibres isolated from the iliofibularis muscle were found to scatter light to different extent when observed in dark field illumination. Fibres from the peripheral zone of the muscle (pale fibres) were found to scatter very little light (Plate 3A), while the majority of the fibres in the dark zone scattered much light (Plate 3B). In the centre of the zone of dark fibres a number of fibres were found which scattered no light (Plate 3D).

The light-scattering properties of single muscle fibres presumably depend on one or more of the structures in the sarcoplasm. In view of the histochemical findings it seems possible that fat droplets and mitochondria either separately or together may contribute to scattered light. It is also possible that the myofibrillar arrangement may be a contributing factor. The myofibrillar arrangement however may be discounted since scattered light can be seen to originate in punctate sources. The conclusion is also supported by some observations of the fine structure of these fibres which show that the myofibrillar arrangement of pale and dark fibres is similar (fibrillar structure). Thus the most likely source of scattered light would seem to be fat droplets and mitochondria, and of these fat droplets are the most probable source owing to their refractivity. If fat droplets alone give rise to scattered light then the sections stained with Sudan Black B are likely to represent a 'map' of the light scattering properties of the fibres in the muscle. If both fat and mitochondria contribute towards light scattering a composite effect will be obtained, the magnitude of which can be estimated from Table 1 since both DPN-diaphorase and succinic dehydrogenase specific granules in the muscle may be taken to represent mitochondria. In any case, the selection of fib

columns 1, 2, 3 and 5 should have been reasonably unambiguous. Fibres of the type shown in the table, column 4, however, may have been included with the fibres of column 3 or with those of column 5.

In these experiments four groups of fibres were selected as shown in Plate 3 ABCD, representing the fibres of Table I, columns 1, 2, (3 and 4) together, and 5 or, the fibres of columns 1, 2, 3, and (4 and 5) together.

End plate morphology

It has been suggested that muscle fibres with different types of end plates also differ in other properties (e.g. Cole 1957, Hess 1960, 1961, Hess and Pilar 1963). The presence of end plates of the en grappe type is a part of the morphological description of amphibian slow muscle fibres (Gray 1956, Hess 1960, Page 1965) while the fast fibres have end plates showing the well known arborizing pattern (e.g. Hess 1960).

End plates were examined on the four groups of fibres as selected by their light scattering properties with the object of detecting any systematic variation in end plate morphology.

Single fibres or small bundles of fibres were dissected out, their light scattering properties were determined and end plates were stained using the thiolacetic acid method for cholinesterases. Plate 3 E, F and G shows such stained end plates. The pictures of Plate 3 are mounted so that the two muscle fibres of each row were from the same region of the muscle and have the same light scattering properties. It is apparent that individual end plates on the pale and dark fibres do show differences in structure, those in the dark region of the muscle being shorter with a tendency in some fibres to be bushy as in Plate 3 G. The pale and dark fibres had one, two or three end plates, two being most common.

The small diameter fibres which scattered no light (Plate 3 D) and which were situated in the centre of the zone of dark fibres failed to show any end plates with the thiolacetic acid method, even though the incubation times were extended until the muscle tendon junctions stained deeply. This result leads to the conclusion that the cholinesterase activity at the end plates of these fibres was very much lower than in any of the other fibres. Nerve terminals on these fibres were successfully stained with the Rongalite-methylene blue method. Ten such fibres, each 6–8 mm in length, were stained and all of them showed terminals of the en grappe type (Plate 3 H). There were usually three widely-spaced endings in each fibre.

The contractile properties of single muscle fibres

The contractions of 33 isolated muscle fibres in response to single and repetitive electrical stimulation were recorded. Acetylcholine was used as a stimulating agent on a number of these fibres. The fibres were classified on the basis of their light scattering properties and their localization in the muscle into four classes. Seven fibres were classified as the type in column 1, Table I, seven as the type in column 2,

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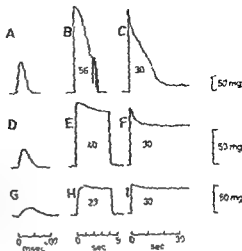
e, v, in column 1 of Table I

D, E, F from a 60 μm fibre of the type in column2 of Table I and G, H, I from a 50 μm fibre of

the type in column 3 of Table I. In B the final

brief decreases in tension are caused by failure

of action potential propagation



thirteen as column 3 type and six as the type in column 5. The latter two classes as explained on p. 60, may have included fibres of the type shown in column 4 of Table I.

Twitch fibres

The fibre was attached to the strain gauge, as described, and its length adjusted so that the peak tension resulting from a single electrical stimulus was maximal. Fig. 2 shows tracings of the mechanical responses of these fibres. The top row is from an experiment with a large fibre from the peripheral zone of the muscle, i.e. a pale fibre (Table I, column 1). The middle row of tracings is from an experiment with a dark fibre of the type in column 2 of Table I. The bottom row of records was obtained from a fibre of the type in column 3 of Table I.

The left hand column in Fig. 2 shows the response of the fibres to a single stimulus and indicates that all these fibres responded with a distinct twitch. The centre column shows the tension resulting from repetitive stimulation at fusion frequency, while the right hand column shows the tension developed during long lasting summation at a frequency of 30 imp/sec. Stimulation of the fibre at a frequency of 30 imp/sec was chosen as a standard testing procedure for all types of fibres because mechanical fatigue could develop rapidly while the electrical impulses in the cell membrane did not drop out as they may do at higher stimulation rates.

Fig. 2 and Table II show that there are differences between the three groups of fibres in the time course of the single twitch, in the fusion frequency and in the duration of maintained tension during repetitive stimulation. The entire collection of fast fibres showed a wide range in the duration of twitch tension. Extreme values of 20 and 80 msec for the contraction time were obtained.

A very striking difference was found between the peripheral pale fibres and central dark fibres in their susceptibility to fatigue. The peripheral fibres

TABLE II Characteristics of the mechanical activity of fast fibres of the types described in table I

Column in Table I	1	2	3
Contraction time of twitch (msec)	29.3 ± 2.3 (7)	37.2 ± 2.0 (7)	53.8 ± 4.5 (13)
Peak to half-decay time of twitch (msec)	34.3 ± 1.3 (7)	34.3 ± 2.5 (7)	56.2 ± 3.5 (13)
Max. tetanic tension (kg/cm ²)	3.3 ± 0.4 (5)	3.1 ± 0.2 (7)	3.1 ± 0.3 (11)
Tetanus to twitch tension ratio	2.0 ± 0.2 (6)	3.0 ± 0.3 (6)	3.7 ± 0.3 (13)
Fusion frequency (imp/sec)	33-60 (5)	33-48 (6)	23-33 (12)
Tension after 30 sec stim. at 30/sec (per cent of initial tension)	3-10 (6)	55-77 (3)	63-93 (11)

The values given are means ± S.E. of mean except for fusion frequency and tension after 30 sec stimulation where the range is given.

Contraction time is given as the time from the beginning of the mechanical response to its peak.

Number in parenthesis indicates number of fibres observed.

quickly when stimulated repetitively (Fig. 2 C) while the dark fibres resisted fatigue (Fig. 2 F, I). The rapid decline of tension occurring in the pale fibres was not due to a failure of propagation of the action potential. This was demonstrated by recording tension and external action currents simultaneously in some of these fibres.

The mean maximum tetanic tension of the whole collection of fast fibres was 3.1 ± 0.2 kg/cm², mean ± S.E. of mean, there being no significant difference between pale and dark fibres in this respect.

Slow fibres

The small clear fibres (Table I, column 5) which were isolated from the centre of the zone of dark fibres responded only with local contractions at the cathode when stimulated electrically. If, with the electrode arrangement shown in Fig. 1 and the fibre at about resting length, a train of stimuli were applied then the resulting tension did not exceed 3 mg or 0.15 kg/cm². However, when a Ringer's solution containing 10⁻⁶ g/ml acetylcholine was applied then the whole fibre contracted rapidly and held a maintained tension of about 4 kg/cm². The fibres relaxed promptly when the acetylcholine was washed away with Ringer's solution, the contractions being repeatable. Acetylcholine caused the other types of muscle fibres (Table I, columns 1, 2 and 3) to give a single twitch only.

Discussion

The evidence presented here supports the broad division of muscle fibres in anuran skeletal muscle into 'fast' and 'slow' groups. It is apparent, however, that the fast group of fibres may be subdivided both on histochemical and functional grounds.

At the present time it would seem to be most useful, in describing the fibre types comprising the iliofibularis muscle of *Xenopus*, to say that there are clearly two broad groups of fast fibres and a group of slow fibres. One group consists of large diameter fibres which, on the histochemical evidence, contain a moderate amount of fat droplets and mitochondria (pale fibres), display a twitch tension of short duration and do not maintain tension on prolonged stimulation (Table II, column 1).

A second group encloses fast fibres containing many fat droplets and mitochondria (dark fibres), with twitch tensions of longer duration and with maintained tension on prolonged stimulation (Table II, columns 2 and 3). That the group of dark fibres might itself be meaningfully subdivided is possible but not demonstrated by the present experiments. The fibres of Table II, columns 2 and 3 might well have been selected from the extremes of a population of fibres with a continuous range of properties. The contractile properties of clear fibres were not studied in sufficient detail to enable one to determine whether or not this group of fibres was completely homogeneous in its properties, but it is certain that none of these fibres propagated an impulse. As pointed out in the Results, it remains a matter of speculation whether the fibres with the properties shown in Table I, column 4 fall into the fast or slow groups.

No fibre that could be said to have properties intermediate between 'fast' and 'slow' was detected, even though more fibres were selected from the vicinity of the slow fibres than from any other part of the muscle (Table I, column 3).

The morphology of the end-plates on the fibre types described above indirectly supports the general conclusions. All fibres which were 'fast' had end plates whose forms may be regarded as falling within the overall morphological variation of 'twitch fibre' end-plates. On the other hand, there does exist a systematic variation in morphology between the end plates on pale and dark fast fibres respectively.

Acetylcholine was applied in some experiments to the various types of fibre. Only the very clear fibres in the centre of the muscle responded with lasting contractions. The situation in the frog appears to be different (Kiesling 1964).

All clear fibres had end-plates of the 'en grappe' type with undetectable cholinesterase activity with the method used. The Rongalite methylene blue method gave very reproducible results and showed, on the average, tree widely spaced clusters of endings on each fibre. An estimate of the depolarization of a fibre of 8 mm length, and a characteristic length of 1 mm, by equidistantly spaced end plates shows that a *point mid way between two end plates will be depolarized by an amount which is about two thirds of the depolarization at an end plate*. Thus the observation of only three clusters of 'en grappe' endings on each clear fibre is not inconsistent with the idea that these fibres function effectively in the absence of a propagated impulse in the cell membrane.

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The Filtration Fractions of Plasma Supplying the Superficial and Deep Venous Drainage Area of the Cat Kidney

By

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Abstract

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In 23 expts the plasma concentrations of protein and inulin were measured in blood collected

In a previous paper (Nissen 1965) the occurrence of different plasma protein concentrations in blood samples obtained from the subcapsular veins (by puncture) and the deep veins (by catheterization) of the cat kidney has been reported. The concentrations were respectively 9 per cent higher and 11 per cent lower than the concentration of arterial blood taken simultaneously.

The discrepancies were interpreted as the result of reabsorption to the deep drainage blood of some of the fluid ultrafiltered from the blood traversing the superficial part of the cortex.

In this paper a method is presented which allows a calculation of the filtration fractions of the plasma flows supplying the two venous drainage areas.

Anatomy

Many mammalian kidneys (including those of man, dog and cat) possess two systems of veins: a deep and a superficial (Fig. 1). The former which drains the inner part of the cortex and the medulla has a course similar to that of the arterial system. However, two essential differences exist: 1) the veins corresponding to the interlobular arteries (the inner cortical veins) begin "blindly"

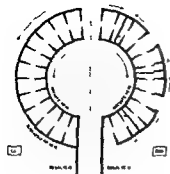


Fig. 1. Left: The two venous systems of the cat kidney. Blood from the superficial cortex to the renal vein passes the outer cortical and the subcapsular veins. Blood passing from the deep cortex (and the medulla — vessels not figured) to the renal vein passes the

the cortex

the arterial system

Theory

The arterial inflow and the venous outflow of plasma of a drainage area in the kidney is called ARPF and VRPF.

The VRPF of an area may differ from that of its ARPF because the rate at which plasma fluid is lost by ultrafiltration in the area may differ from that by which fluid is regained by reabsorption.

The filtration fraction (FF) is defined as the filtration rate (F) divided by arterial plasma flow. $FF = F/ARPF$. A_1 is the plasma concentration of inulin in arterial blood, V_1 the plasma concentration of inulin in the renal venous blood from the area in question. $f_w (= 1.05)$ is a factor correcting a plasma concentration to the concentration in plasma water.

For the area in question the inflow of inulin in the arterial plasma minus the amount of inulin filtered is equal to the amount drained away by the venous plasma flow.

$$ARPF \cdot A_1 - (FF \cdot ARPF) \cdot (A_1 / f_w) = VRPF \cdot V_1$$

$$\text{or } 1 - FF \cdot f_w = \frac{VRPF \cdot V_1}{ARPF \cdot A_1} \quad (1)$$

¹ This was tested experimentally in the cat. A polyethylene catheter, the same type as used in the remaining experiments, was inserted through one of the two branches of the renal vein into the deep veins. The pressure was 9–14 mm Hg (as compared to 4–5 mm Hg in the caval vein). When a ligature was tightened around the branch with the catheter the pressure only rose 5 mm Hg.

As protein (P) for all practical purposes is not filtered in the glomeruli we obtain

$$\begin{aligned} \text{ARPF} \cdot A_p &= \text{VRPF} \cdot V_p \\ \text{or } \frac{\text{VRPF}}{\text{ARPF}} &= \frac{A_p}{V_p} \end{aligned} \quad (2)$$

Inserting 2 in 1 yields

$$\text{FF} = \frac{1}{f_w} \left(1 - \frac{V_i}{A} \cdot \frac{A_p}{V_p} \right) = \frac{1}{f_w} \cdot \frac{\frac{V_p}{A_p} - \frac{V_i}{A_i}}{\frac{V_p}{A_p}} \quad (3)$$

V_x/A_x is called the *fractional concentration* of X

Discussion In setting up formula 1 it is presumed that *inulin* is completely filterable in the glomerulus and that it is neither synthesized, destroyed, excreted or reabsorbed by the tubule. These "classical" requirements to inulin are discussed by Smith (1951). In some of my experiments both inulin and creatinine were used as "indicator" substance to compare the results of creatinine with those of inulin.

I have investigated this claim using an improved technique. The results constitute a part of the work presented in this report.

The formulas 1 and 2 are only correct if a steady state exists for the renal interstitial fluid volumes and concentrations.

care was taken to maintain constant arterial pressure and constant arterial concentrations of inulin (and creatinine) during the experiments.

The renal lymph flow is small, approximately half the normal urine flow (Mayerson 1963) which is again roughly 0.2 per cent of the plasma flow. Furthermore, this fluid is not inulin- and protein-free according to Kaplan, Friedman and Kruger (1943) and Mayerson (1963); the protein and inulin concentrations are approximately 0.5 and 0.7 times the arterial concentrations. Both facts indicate that the lymph flow constitutes only a minor source of error in the experiments. No attempts were made to correct this error.

Method

The experiments were performed on 26 cats averaging 4.5 kg in weight (range 3.0–6.8). They were anesthetized with pentobarbital 40 mg/kg i.p. plus supplementary doses i.v. or with chloralose 60–90 mg/kg i.p. The animals, who in the days prior to the experiment were fed liberally with meat and fish, were fasted for 14–18 hours prior to the experiments. These were carried out with the animals placed in the supine position on a heated table. The arterial blood pressure was measured in a carotid or femoral artery. Approximately 25 mg heparin was injected just before taking the blood samples.

Collection of arterial and mixed renal venous blood. Degradation of protein in the kidney. 3 experiments (in chloralose anesthesia). The arterial blood was sampled from a carotid artery. In order to ensure that the blood from the two venous drainage areas was well mixed before collection, the blood was first led through 6–8 cm of a PVC tube through which 4–6 pins had been thrust to produce turbulence. The tube (i.d. 3–4 mm) connected the inferior caval vein just distal to the kidneys with the same vein proximal to these. This bypass produced minimal stasis of the kidneys. While inserting the PVC tube the renal blood was temporarily led through a side branch of the tube to a jugular vein to avoid any interruption of the renal blood flow. The blood from the inferior extremities was led to the other jugular vein.

The two samples were collected simultaneously by a multi-tube roller pump (Desaga) at rates of 0.15–0.30 ml per minute.

2 × 3.5 ml were collected. During each sampling period the diuresis and the renal plasma flow were determined, thus the rise of the fractional protein concentration of mixed renal venous plasma caused by loss of urine could be allowed for. RPF was measured as the excretion rate $\frac{PAH}{V_{PAH}}$ divided by $\frac{PAH}{V_{PAH}}$, where V_{PAH} in this case is the PAH concentration in the mixed renal venous plasma.

100

To facilitate the insertion a 0.5–1.0 mm long incision was made in the tough fibrous capsule

ureter, in 4 expts the ureter was catheterized to keep the urine clear of the slight hemorrhage arising from the divided ureter.

In all the experiments a priming dose followed by a constant infusion (0.4–0.5 ml/min) of inulin in 0.9 per cent saline was given in a jugular vein. In addition in some experiments creatinine, glucose, phloridzin, paminohippurate, sodium or potassium-phosphate was administered. The results from a few periods in which an osmotic diuresis exceeding 0.2 ml/min from the experimental kidney was provoked are excluded; the subject of osmotic diuresis is treated in a subsequent paper. Small doses of donor blood were given if the blood pressure showed a tendency to fall.

At the end of the experiment the kidney was inspected to determine the position of the deep catheter and to disclose any lesion of the kidney tissue made by the catheter. A lesion in the superficial cortex was observed only once (the corresponding data were discarded); in the deep regions of the kidney no lesions were observed.

Analyses. The protein was determined in duplicate by a biuret reaction (Cornall, Bardswill and Day 1919) using 100 μ l plasma for each determination. The standard deviation (SD) of the single determination determined from duplicates ($SD = \sqrt{\frac{\sum d^2}{2n}}$) was 0.6 per cent of the mean ($2n = 10$).

All deproteinizations were made in duplicate; two determinations were made from each deproteinized sample.

Inulin was analyzed by the method of Bojesen (1952) using 1/5 the volume of the original procedure. 100 μ l plasma was used for each deproteinization. The coefficient of variation of the single deproteinized sample was 0.8 per cent ($2n = 156$). Glucose brought about an extinction of 1 per cent of that of inulin; a correction for this was applied with low inulin concentrations or high glucose concentrations or both.

Creatinine was analyzed according to Peters (1912) modification of Jaffe's reaction using 500 μ l plasma for each deproteinization. The coefficient of variation was 1.0 per cent ($2n = 152$). The plasma recovery was determined for each analytical batch; normally it was between 87 and 90 per cent (range 83–100).

Glucose was analyzed by an enzymatic method (Bergmeyer 1963) using 100 μ l plasma per deproteinization. The coefficient of variation was 0.6 per cent ($2n = 151$). Fluoride admixture to the blood samples was not used as the salt would produce osmotic reduction of the red cell volume thus producing a plasma dilution. To minimize the ensuing degradation of glucose the samples were centrifuged immediately after the period.

The coefficient of variation due to the analytical error) on the final expression FF ($\frac{V_F}{V_P} \cdot \frac{V_1}{V_2}$) (cf. appendix) was of a reasonably low value (about 4 per cent) firstly because the determinations were all was performed in duplicates; this reduces the coefficient of variation of the protein and inulin determinations by the factor $1/\sqrt{2}$ in the double determinations. Secondly because the venous and arterial plasma concentrations used in the FF expression were always derived from the same analytical batch (and thus allowing the standard deviation to be calculated from the duplicates). Calculation

The fractional SD of the expression

$$FF = \frac{V_F}{V_P} \cdot \frac{V_1}{V_2} = 1 + \frac{V_1}{V_P} \cdot \frac{V_2}{V_F}$$

may be calculated using 0.30 as a representative value for this quantity and 1.00 as a suitable coefficient of variation of both analyses.

The fractional SD of V_I/A_I and V_P/A_P is

$$\sqrt{\left(\frac{0.008}{\sqrt{2}}\right)^2 + \left(\frac{0.008}{\sqrt{2}}\right)^2} = 0.008$$

The fractional SD of $(V_I/A_I) - (V_P/A_P)$ is

$$\sqrt{(0.008)^2 + (0.008)^2} = 0.009 \sqrt{2}$$

The "absolute" SD of $1 - (V_I/A_I) - (V_P/A_P)$ is

$$0.70 (0.009 \sqrt{2})$$

and the fractional SD of the same expression

$$\frac{0.70 (0.009 \sqrt{2})}{0.30} = 0.026$$

In the final ratio FF_D/FF_S the factor $1/f_w$ cancels out. So the fractional SD of FF_D/FF_S will be

$$\sqrt{(0.026)^2 + (0.026)^2} = 0.026 \sqrt{2} = 0.037$$

Results

Collection of arterial and mixed renal venous blood. Degradation of protein in the kidney. The fractional protein concentration (V_P/A_P) was determined in 32 periods of 3 experiments. In 23 the blood pressure varied at the most 20 mm Hg within the period, and the blood pressure level was evidently above the lower limit of the autoregulatory range of the renal blood flow. When these conditions were not fulfilled the number is given in brackets.

First experiment 1.005, 1.007, 0.993, 1.001, 1.003, 0.995, 0.989, (0.986), (0.996), (0.972)

Second experiment 0.994, 0.989, 0.976, 0.994, 0.987, 0.982, (0.993), (0.993), (1.002), (1.015), (1.023), (0.990)

Third experiment 1.003, 0.998, 1.002, 0.997, 1.001, 1.000, 0.990, 0.987, 1.003, 0.997

The average figure for the 23 periods was 0.995. The correction for the loss of urine, to be subtracted from the fractional concentrations, averaged 0.002 in the 3 experiments.

Collection of arterial blood and of venous blood from the two drainage areas. 1. Comparison between the handling of inulin and creatinine in the kidney. The results are derived from 39 periods in 10 experiments. The average clearance of inulin for the experimental kidney was 7.6 ml/min (range 2.3—13.8). The average creatinine to inulin clearance ratio was 0.94, SD 0.10 ($n = 37$). It is significantly lower than 1.00 ($p < 0.001$, Student's t).

A reasonable agreement existed between the creatinine to inulin clearance ratios and the ratios between the creatinine extraction fractions and the inulin extraction fractions for the two drainage areas [$1 - (V_I/A_I) - 1 - (V_P/A_P)$]. Thus the mean ratio for the superficial area was 0.94, SD 0.03 ($n = 39$) and for the deep area 0.93, SD 0.07 ($n = 37$). A value of 2.3 was omitted in the calculation of the former mean. The means are significantly lower than 1.00 ($p < 0.001$).

The range of the arterial plasma concentrations of inulin was 11—83 mg/100 ml (mean 39). The range of the plasma concentrations was 27—89 mg/100 ml (mean 48).

2 *The filtered fractions* FF_S and FF_D of the two drainage areas, calculated from 75 periods in 23 experiments, are presented in Fig 2a. The average FF_S was 0.34. For FF_S values in the range 0.1–0.2 the mean FF_D/FF_S ratio was 0.7. For FF_S values in the ranges 0.2–0.3, 0.3–0.4 and 0.4–0.5 the corresponding mean FF_D/FF_S ratios were 0.80 ± 0.02 (SE), 0.86 ± 0.03 (SE) and 0.88 ± 0.02 (SE), the 3 last means were significantly lower than 1.00 ($p < 0.001$). The average FF_D/FF_S for all the periods was 0.85.

In 10 experiments (43 periods) both creatinine and inulin was infused. The FF_D/FF_S calculations based on the creatinine (and protein) concentration yielded an average value of 0.74 (Fig 2b).

Discussion

Degradation of protein in the kidney The experiments showed an average fractional (venous/arterial) plasma protein concentration very near to 1.000 (0.995). This finding is in agreement with Latham and Benjamin's (1956) observation that no Δ differences for protein could be demonstrated in the dog kidney. Considering the magnitude of the renal plasma flow the results are consonant with the facts that the degradation rate of plasma protein in the whole organism is only about 0.3 g/kg \times day (cf. for example Jarnum 1963) — and that the degradation occurs only to a minor extent (below 10 per cent) in the kidneys (Katz, Rosenfeld and Sellers 1960; Katz, Sellers and Bonorris 1964). Thus protein is not degraded in the kidney to such an extent that it invalidates the use of the fractional protein concentration in the formulas.

The reason for renal Δ differences of 1–5 per cent of the arterial concentration measured by other authors (Gerbi 1951; Harms *et al.* 1962) is probably a dilution of the renal capillary blood by interstitial fluid. The cause of dilution is the withdrawal of rather large blood samples, resulting in a fall in arterial and renal venous pressure (blood is collected from the renal vein). Renal vasoconstriction may also cause a fluid displacement from the renal interstitial spaces to the capillaries. A dilution was obtained in dogs in exaggerated form by Heimbarg and Ochwaldt (1961) by lowering of the blood pressure in the abdominal aorta or by i.v. injection of adrenalin.

Comparison between the renal handling of inulin and creatinine One group of authors (cf. Smith 1951) has obtained rather good agreement between inulin and creatinine clearances in dogs and cats. Agreement, however, was not obtained by Eggleton and Habib (1951) and Peters (1963). With low inulin concentrations Eggleton and Habib (1951) found that the inulin clearance was the larger; with higher concentrations the creatinine clearance was the larger. Opposite results were obtained by Peters.

Ladd, Liddle and Gagnon (1956) obtained a mean creatinine to inulin clearance ratio, significantly below 1.00 (0.94 ± 0.03 SD) in the dog. They concluded that transcellular back diffusion of creatinine takes place (Levinsky and Berliner 1959).

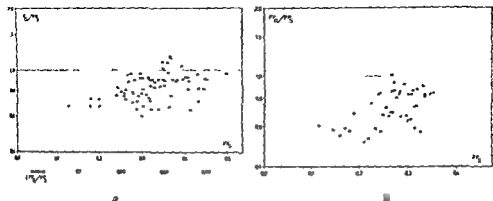


Fig. 2. (a) FF_D/FF_S vs FF_S . (b) FF_D/FF_S vs FF_S . Symbols as in Fig. 2a.

age area

Fig. 2b Results based on the fractional creatinine and protein concentrations. Symbols as in Fig. 2a

present evidence for back diffusion of creatinine from the ureter and suggest that this process is one of the reasons why a ratio below 1.00 is obtained. An average ratio of 0.94 has been found in the present work.

In microinjection experiments in the rat Bauman *et al.* (1965), Maude *et al.* (1965) and Gottschalk, Morel and Mylle (1965) have shown that inulin is not reabsorbed in the tubules.

It is reasonable to presume that a creatinine to inulin clearance ratio below 1.00 is the result of back diffusion of the smaller creatinine molecule, and to rely on the assumption that inulin clearance is a measure of the glomerular filtration rate.

The filtration fractions (FF_S and FF_D) The FF_S (S for superficial drainage area) averaged 0.34, using inulin as the indicator. The average FF_D/FF_S calculated for various ranges of FF_S values was consistently below 1.00. A ratio of 0.85 may be used as a representative value (cf. results and Fig. 2a). FF_D/FF_S values below 1.00 were also obtained with creatinine as the indicator substance (Fig. 2b).

The question may now be raised: is the difference between the filtration fractions of the two areas explainable by distinct differences in the structure of the two areas?

The FF of the plasma entering a glomerulus is a function of the colloid osmotic pressure of the plasma, and the hydrodynamic resistances of the afferent and efferent vessels and of the membrane of the glomerular capillaries; also the rate of reabsorption of fluid by the proximal tubule is probably an important factor (Bojesen 1954, Kruhoffer 1960).

When vascular by-passes of the glomeruli are present in an area, the FF of the area will not be determined solely by the IT of the plasma entering the glomeruli, but the fraction of plasma traversing the factor

Now, assuming that the FF of plasma entering the glomerular capillaries are identical in superficial and deep glomeruli but that some plasma by-passes the deep glomerular capillaries we may use the equation

$$F_D / (ARPF_D - ARPF_{BP}) = FF_S \quad (I)$$

where $ARPF_{BP}$ is the by-pass flow. The results of the experiments yielded (using the representative value of 0.85 for FF_D/FF_S)

$$F_D / ARPF_D \approx 0.85 FF_S \quad (II)$$

II divided by I gives

$$ARPF_{BP} / ARPF_D - 1 - 0.85 = 0.15$$

Thus the by-pass flow should constitute a fraction of 0.15 of the plasma flow supplying the deep area. This is approximately 15 per cent = 11 per cent of the plasma flow supplying the whole kidney as the ratio $ARPF_D / ARPF_S$ is about 1.1 and therefore $ARPF_{BP} / (ARPF_D + ARPF_S) = 0.15 / 1.1$. The figure 1 is derived from the two facts: 1) that the fractional protein concentration (V_p/A_p) of the superficial and deep drainage plasma averages 1.09 and 0.89 (Nissen 1963) and 2) that the mixing of these plasma streams must yield plasma (in the renal vein) with a fractional protein concentration very near to 1.00. The ratio between the two venous plasma flows D/S is thus $1.09/1.00 = 1.00/0.89$, and the ratio between the arterial flows $1.09/1.00 = 0.89/1.00 = 0.89/1.09$.

Various by-pass vessels situated outside the renal corpuscles of the deep cortex have been described: these are the coiled arteries of the renal sinus, the arteriolae rectae verae and the Isaacs-Ludwig arterioles. The first vessels are aglomerular branches from the renal interlobar or arcuate arteries which partly end in the capillary plexus of the calyculi mucosa (Klugelgen and Passarge 1960) and partly supplement the vasa recta system supplying blood to the papillary zone (Baker 1959). The arteriolae rectae verae are aglomerular types of arteriolae rectae spuriae, the latter forming the principal supply to the medulla. The Isaacs-Ludwig arterioles are branches of normal afferent arterioles ending in the peritubular capillary plexus or as arteriolae rectae verae. For details the reader is referred to Selkurt (1963), Klugelgen *et al.* (1959), and Smith (1951). However, these three kinds of aglomerular vessels of the normal kidney are rare, so rare that it is difficult to imagine that about 11 per cent of the renal plasma flow passes through them.

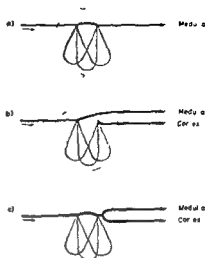
Are there then — inside the corpuscles of the deep cortex — by-pass vessels of greater capacity? The works of Zlabek (1957), Ljungqvist and Lagergren (1962) and Granberg, Lagergren and Ljungqvist (1964) offer information on this subject. Zlabek (1957) made polymethylmetacrylate casts of the vessels of 3 human kidneys, ages 10, 18 and 38 years. Juxtamedullary glomeruli were defined as those possessing an arteriola recta spuria. Out of 62 normal (non-degenerated) juxtamedullary glomeruli, 56 possessed a wide vasa efferentia (of about the same width as the vasa afferentia). In 26 out of these 56 glomeruli and in none of the remaining, a wide "short-circuit" between the afferent and the efferent arteriole was clearly visible on the surface of the glomeruli. In the remaining 30 glomeruli a short-circuit could not be observed.

Fig 3 Sketch of proposed anatomy of juxtamedullary glomeruli

a) Juxtamedullary glomerulus with Zlabek's short circuit and one efferent arteriole

b) Juxtamedullary glomerulus with two efferent arterioles

c) The blood to the medulla passes the short



low

by direct inspection, but in two of these a short circuit was observed on closer examination, the author believes that all juxtamedullary glomeruli with a wide vas efferens possess this short circuit placed superficially or deeply in the tuft. The observation, that the efferent arterioles of the juxtamedullary glomeruli are wider than those of the rest of the glomeruli, was already made by Trueta *et al* (1947). Christensen (1952) could not affirm the observation, however it was reaffirmed by Ljungqvist and Lagergren (1962) and Zlabek (1957).

Ljungqvist and Lagergren (1962) investigated the anatomy of the renal vessels by stereo-micro-angiographic and histologic techniques. They found that degeneration of the juxtamedullary glomeruli owing to age produces atrophy of only the capillary tuft while the continuity between the afferent and efferent arterioles is retained and an aglomerular arteriole with an arteriola recta vera is formed. In partly degenerated, juxtamedullary glomeruli the short circuit may be seen distinctly. Degeneration of other glomeruli, however, involves atrophy of the efferent vessel, the glomerular tuft and the afferent vessel, in one stage of the process the latter is seen as a thin, blindly ending twig. Ljungqvist and Lagergren (1962) believed that the differences between the degeneration forms are caused by the normally occurring short circuits described by Zlabek (1957).

Zlabek's (1957) short circuits, the arteriolae rectae verae and the coiled arteries supply the medulla. These vessels would probably be adequate anatomical counterparts to the low IF_p/IF_s , since a plasma flow through them of approximately 6 per cent of the total renal plasma flow would not — a priori — be unreasonable. A medullary flow of 6–8 per cent has been found in dogs by other authors (Deeten, Brechtelsbauer and Kramer 1964).

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The Relation between Reabsorption Rate and Filtration Rate in the Superficial and Deep Venous Drainage Area of the Cat Kidney

By

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Abstract

NISSEN, O. I. *The relation between reabsorption rate and filtration rate in the superficial and deep venous drainage area of the cat kidney* Acta physiol. scand 1966 68 286—294

In 23 expts in cats the plasma concentrations of protein, inulin and glucose have been measured in blood collected simultaneously from an artery and from subcapsular and deep veins of the kidney. For each venous drainage area it is possible to calculate the ratio reabsorption rate/filtration

complicated pattern emerges for the drainage of the reabsorbate from different tubule segments to the vein systems

In a previous paper (Nissen 1966) the author presented a method for the determination of the filtration fractions of the plasma supplying the two venous drainage areas of the cat kidney. The calculations were for each area based upon the concentrations of inulin and protein in the venous plasma relative to those of the arterial plasma.

In the present paper a formula is given that expresses the rate of tubular reabsorption as a fraction of the glomerular ultrafiltration in the area. Another equation gives the ratio reabsorbed to filtered amount of a substance in an area. The renal handling of glucose is studied on this basis.

The drainage of the reabsorbed fluid from different parts of the nephrons to the two vein systems will be considered in some detail.

Theory

Calculation of the ratio reabsorption rate/filtration rate in a drainage area The filtration fraction (FF) of the plasma supplying a drainage area is calculated as (Nissen 1966)

$$FF = \frac{1}{f_h} \cdot \frac{V_p/A_p - V_i/A_i}{V_p/A_p} \quad (1)$$

A_p is the concentration of protein in arterial plasma and V_p the concentration of protein in plasma from the area in question A_i and V_i are the corresponding concentrations of inulin f_h (≈ 1.0) is a factor correcting plasma concentrations to concentrations in plasma water

As the lymph flow is very small in comparison with the plasma flow the venous plasma flow from an area (VRPF) must be equal to the rate of plasma flowing into the area (ARPF) minus the filtration rate (F) and plus the reabsorption rate (R)

$$ARPF - F + R = VRPF$$

or $1 - F/ARPF + R/ARPF = VRPF/ARPF$ 2)

$F/ARPF$ is the filtration fraction (FF), $R/ARPF = RF$ is the ratio between the rate of fluid reabsorption in the area and its arterial plasma flow

As the glomerular capillaries are practically proteintight, we get $ARPF \cdot A_p = VRPF \cdot V_p$ or $VRPF/ARPF = A_p/V_p$

Inserting 1 and 3 in 2 and isolating RF yields

$$RF = \frac{1}{f_h} \cdot \frac{f_h + V_p/A_p (1 - f_h) - V_i/A_i}{V_p/A_p} \quad (4)$$

$(1 - f_h)$ amounts to ≈ 0.05 , and V_p/A_p is in the range of 0.85 to 1.15 The rest of the numerator $(f_h - V_i/A_i)$ generally has a magnitude of about 0.30 From this it follows that only a small percentual error of RF will be introduced if $V_p \cdot A_p$ is substituted by 1 in the numerator

The simplified formula is

$$RF = \frac{1}{f_h} \cdot \frac{1 - V_i/A_i}{V_p \cdot A_p} \quad (5)$$

The ratio between the RF and FF of an area equals the ratio between reabsorption rate and filtration rate in the area

$$RF/FF = R/F = \frac{1 - V_i/A_i}{V_p \cdot A_p - V_i \cdot A_i} \quad (6)$$

Calculation of the ratio reabsorbed amount to filtered amount of a freely filtrable non-electrolyte

The amount of the substance G for glucose carried to the drainage area by the arterial plasma, is $VRPF \cdot A_G$ The filtered amount is $F \cdot f_h \cdot A_G$ and the reabsorbed amount $R \cdot R_G$, where R_G is the average concentration of glucose in the reabsorbate

The amount drained away from the area is $VRPF \cdot V_G$ Provided formation and conversion of glucose in the area is negligible

$$ARPF \cdot A_G - F \cdot f_h \cdot A_G = R \cdot R_G = VRPF \cdot V_G$$

Isolating the unknown (reabsorbed amount/filtered amount) and inserting formula 1 and 3 yields:

$$\frac{R \cdot R_G}{\Gamma \cdot f_w \cdot A_G} = \frac{(VRPF/ARPF) \cdot (V_G/A_G) + (\Gamma/ARPF) \cdot f_w - 1}{(\Gamma/ARPF) \cdot f_w}$$

$$\text{or } \frac{R \cdot R_G}{\Gamma \cdot f_w \cdot A_G} = \frac{V_G/A_G - V_I/A_I}{V_P/A_P - V_I/A_I} \quad (7)$$

Discussion The simplification of formula 4 introduces a systematic error in formula 5 and 6. In the superficial venous plasma the fractional concentration of protein (V_P/AP) and of inulin (V_I/A_I) amounts to about 1.10 and 0.70, respectively. Therefore the RF of the superficial area is overestimated by 1–2 per cent by the simplification. Conversely, when the deep venous plasma has a fractional protein and inulin concentration of about 0.90 and 0.65, the RF will be underestimated by 1(–2) per cent.

However, an experimental fact tends to compensate for these errors. In the superficial venous plasma the concentration of sodium with matching anions is 1(–2) per cent less than in arterial

cent

The "simplification error" and the "osmotic error" partly cancel each other reducing the systematic error of the RF to 0.5–1 per cent. (Neither formula 1 nor 7 is affected by the two errors because the simplification is not introduced and because the osmotic error changes the numerator and the denominator by the same factor since the red cell membrane in the cat is impermeable for protein, inulin and glucose (Kozava 1914).)

For a further discussion of methodological errors, see Nissen 1966.

Method

The results reported in this study are based on a series of experiments briefly described in two

in Nissen 1966

In addition to inulin 0.9 per cent NaCl, creatinine or glucose was also infused i.v. in some experiments. In other experiments the animal was loaded with p-aminohippurate, sodium phosphate or phloridzin. The results from a few periods in which an osmotic diuresis exceeding 0.2 ml/min from the experimental kidney was provoked are excluded. The subject of osmotic diuresis will be treated in a subsequent paper.

Results

The fractional concentrations from a single experiment of inulin, protein and glucose in blood collected from superficial and deep veins are given in Fig. 1. Below the abscissa the results are tabulated. A rise (29–51 mg per cent) in the level of the plasma inulin concentration was produced between period 3 and 4. R/F (formula 6 in "theory") refers to the ratio between distances on the graph, e.g. for period one R_3/F_3 equals a/b . The fractional concentrations of inulin and protein in the superficial drainage plasma were higher than the respective fractional concentrations in the deep plasma. IF_3 was greater than IF_D (Nissen 1966). Furthermore RF_3/IF_3 was always below 1 and RF_D/IF_D above 1. This fact together with the observation

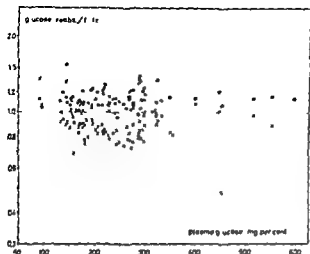


Fig. 3. Abscissa: Concentration of glucose in arterial plasma. Ordinate: \bullet represents the ratio reabsorbed amount/filtered amount of glucose in the superficial drainage area; \times represents the response ratio in the deep area.

that the ratio reabsorbed amount to filtered amount of glucose is generally above 1 for the superficial area and below 1 for the deep area, will be discussed below.

The rise in the plasma inulin level did not produce any changes in the results.

$R_F/F_F = R/F$. In Fig. 2a all the R_F/F_F values are given using inulin as the indicator substance (23 expts., 78 periods). Essentially the same results were obtained using creatinine — but fewer periods (46) were made with this substance (10 expts. — Fig. 2b).

With inulin as the indicator the average values for R_F/F_F in the $I_{1,1}$ ranges 0.1–0.2, 0.2–0.3, 0.3–0.4 and 0.4–0.5 were 0.7, 0.79, 0.79 and 0.77; the standard error (S.E.) of the last three means was 0.01. The average values for R_F/F_F in the corresponding $I_{1,1}$ ranges were 1.5, 1.37, 1.23 and 1.33. The S.E. of the last three means were 0.03, 0.02 and 0.03. All the R_F/F_F and R_F/F_F ratios where S.E. is stated are below 1 and above 1 for $p < 0.001$ Student's t .

Glucose. In 76 periods of 19 expts. the glucose threshold was not exceeded; the total excreted/filtered amount of glucose was below 0.01.

In these periods the ratio reabsorbed/filtered amount for the superficial area averaged 1.13, S.E. = 0.01. For the deep area the average value was 0.81, S.E. = 0.01. The two means were different from 1.00, $p < 0.001$ Fig. 3.

Discussion

Two major findings will be discussed. Firstly, that the reabsorption rate is lower than the filtration rate in the superficial drainage area and that the opposite applies to the deep area (Fig. 1 and 2). Secondly, the paradoxical finding that the ratio reabsorbed/filtered amount of glucose is above 1 in the superficial area and below 1 in the deep area.

As an introduction a short summary of renal anatomy will be given. The borderline separating the two drainage areas is indicated in Fig. 1 by the upper thin horizontal

line. The border of the medulla is marked by a thick line. All nephrons in the kidney are symbolized by two nephrons, the corpusculum of one lying in the superficial cortex, and that of the other in the deep cortex. The pars convoluta of the proximal tubule is horizontally depicted (the "loop" of the proximal tubule of the deep glomerulus is referred to later in the paper). From the end of the convoluted tubule the pars recta stretches to the medulla. The pars recta becomes the thin limb of the loop of Henle at the transition between the outer and inner strafe of the outer medullary zone.

All the loops of the cat kidney are long, viz. they reach into the inner medulla. Where the latter borders the outer medulla the distal thin limb continues in the thick limb of the loop of Henle. The thick limb passes out into the cortex and after running a course close to its corpuscle, it is gradually converted into an initial and proper collecting tubule. The majority of these transitions are situated in the most superficial part of the cortex — this applies even to nephrons with deeply lying corpuscles. Thus most of the nephrons possess not only a "medullary loop", but also a "cortex loop". From an anatomical aspect considerable importance has been attached to the description of these cortex loops in different species (Peter 1927, v. Sollen-dorff 1930, Sperber 1944).

From this provisional scheme it is reasonable to assume that the fluid ultrafiltered from the blood passing the superficial area is partly reabsorbed into this blood and partly (in the loops of Henle and the collecting tubules) to that traversing the deep area, further that the fluid ultrafiltered from the deep blood is reabsorbed partly into the deep blood (in the proximal tubules, loops of Henle, and collecting tubules) and partly into the superficial blood (by the cortex loops and the upper part of the collecting tubules). It may be presumed that the fluid volume ultrafiltered superficially and reabsorbed profoundly exceeds the volume ultrafiltered profoundly and reabsorbed superficially, as the proximal tubules constitute a considerable part of the "medullary" loops, the fewer and shorter cortex loops consist of distal tubules and collecting tubules. This view is consistent with the finding of a R/F ratio (cf. appendix) below 1 in the superficial area and above 1 in the deep area.

However, the model as described so far cannot explain the fact that more glucose is reabsorbed than filtered in the superficial area, and vice versa in the deep area (Fig. 1 and 3) viz. if the view that glucose is reabsorbed merely in the proximal tubules holds good then the ratio reabsorbed amount/filtered amount of glucose in the superficial area should be 1 or less. The value 1 would occur if the reabsorption of the glucose filtered in the superficial area was completed in those parts of the proximal tubules, which are surrounded by blood drained by the superficial veins. In this case the ratio reabsorbed amount/filtered amount of glucose in the deep area would adopt its minimal value which is also 1 as long as Γ_{glucose} is not exceeded.

It seems unlikely that a conversion or synthesis of glucose quantitatively is responsible for the considerable deviations of the ratio from 1. From a qualitative point of view such an explanation seems equally unlikely because it would be necessary

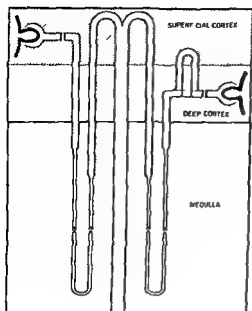


Fig. 4. Model of the nephron population. The nephrons are represented by one symbolizing those originating in the superficial drainage area, another those originating in the deep area.

to assume that a conversion took place in one area (the deep one), while a synthesis occurred simultaneously in the other. The key to the problem may lie in the localization of the tubules belonging to glomeruli situated along the border, separating the two drainage areas. Anatomists (Peter 1927, v. Mollendorff 1930) often emphasize that the glomerulus of the mammalian kidney is situated profoundly (centrally) to the attached ball of the proximal tubule. From the descriptions and drawings of these balls of the cat (Peter 1927) it may be deduced that the proximal tubules of the middle part of the cortex often have their loops lying 1.0–1.5 mm in the superficial direction from the attached corpusculum. The total thickness of cortex is 4–6 mm.

Now it can be seen that a certain number of border-nephrons are filled with a fluid ultrafiltered from the plasma that supplies the deep drainage area while the proximal tubules of these nephrons are partly bathed in plasma, that is drained by the superficial veins. The existence of these nephrons is indicated in Fig. 4 by a loop of the proximal tubule extending into the superficial area.

A ratio between reabsorbed and filtered amounts of glucose above 1 for the superficial area and below 1 for the deep drainage area may now be explained viz. it results from a reabsorption into the superficial plasma of some of the glucose ultrafiltered from the deep plasma in the glomeruli of the border-nephrons. If any glucose was reabsorbed in the cortex loop, these formations might also account — at least partially — for the deviations of the ratio from 1.

The intratubular transit of glucose across the border averages 13 per cent of the amount of glucose filtered in the superficial area ($\text{reabs. G}/\text{filtr. G} = 1/13$). The great majority of these proximal loops of the borderline were probably saturated in

respect to glucose transport as the plasma glucose concentrations were high, although they did not exceed the plasma threshold (Fig. 3). Further it is probable that some proportionality exists between the glucose transport capacity and the rate of fluid reabsorption of the proximal tubule mass. Accepting this it may be suggested that a reabsorbate volume amounting to roughly 10 per cent of the superficial ultrafiltrate is transported from the deep to the superficial area by the border-nephrons.

This estimate makes it possible to give a rough quantitative description of the handling of the volume, ultrafiltered in the superficial drainage area.

Assuming the ratio R_2/F_2 to be 0.78 (p. 5), per 100 ml ultrafiltrate of the superficial area, 78 ml are reabsorbed by the tubules of this area. About 10 ml originate from the proximal loops of the border nephrons and thus they are ultrafiltered by the deep glomeruli. The rest, 68 ml, are proximal reabsorbate (ultrafiltered superficially) plus distal reabsorbate from the cortex loops and the collecting ducts situated in the superficial area (Fig. 4). Presuming the latter reabsorbate volume to be 5 ml (cf. for example Kruhoffer 1960, p. 250) it will be seen that approximately $68 - 5 = 63$ ml of the 100 ml filtered are reabsorbed by the proximal tubules of the superficial area. The remaining 37 ml, are reabsorbed by the loops of Henle by the rest of the distal tubules, and by the collecting ducts; a minor part is excreted as urine.

On the kidney surface 55 to 60 per cent of the proximal tubule length was accessible in the micropuncture experiments of Walker *et al.* (1941) in the rat and Clapp, Watson and Berliner 1963 in the dog. From these works and from that of Peter (1927) it may be worked out that about 3/4 of the length of the proximal tubules of superficial origin lie in the superficial drainage area, assuming a borderline in the middle of cortex. If the view set forth by Walker *et al.* (1941) that the reabsorbed amount of fluid per length unit of proximal tubules is constant throughout the tubules, then $100 - (63 \cdot 4/3) = 16$ ml would be handled by the distal tubules.

Appendix

Definition of Symbols

- D and S — Subscripts referring to deep and superficial renal venous drainage area
 ARPF — Arterial renal plasma flow supplying a venous drainage area
 VRPF — Venous renal plasma flow leaving a venous drainage area
 F — Volume ultrafiltered per time unit in a venous drainage area
 R — Volume reabsorbed by the tubules per time unit in a venous drainage area
 FF — F/ARPF = filtration fraction in a drainage area
 χ — The plasma concentration of a substance χ in arterial blood
 χ_v — The plasma concentration of a substance χ in venous blood from a drainage area
 χ_v/χ — The fractional concentration $f\chi$
 I, P, G — Subscripts referring to interstitial, peritubular and glomerular
 $f\chi$ — $f\chi$ is a factor which corrects plasma concentration of a substance to the concentration of the substance in the plasma water

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The Action of Adrenaline in Cardiac Muscle II.

Effect on Oxygen Consumption in the Asystolic Perfused Rat Heart

By

ANTON HALGE and IVAR OYE

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Abstract

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Oxygen consumption of isolated rat hearts at 37°C. was found to be 0.36 ml/min per g

which directly or indirectly are stimulated by adrenaline

Adrenaline increases the oxygen consumption of cardiac muscle (Barcroft and Dixon 1907). The increase can only partially be accounted for by an increased stroke work and the term "oxygen waste" has been introduced to describe the excess increase in oxygen uptake (Raab 1962). The 'oxygen waste' might reflect either an increase in contractile activity which does not result in increased pump work ('internal work') or a direct effect of adrenaline on cardiac metabolism, or both. An increase in cardiac oxygen consumption after adrenaline during experimental asystolia in the perfused rat heart has been described by Challoner and Steinberg (1965) and by Hauge and Oye (1966). In the present work the calorogenic effect of adrenaline has been studied under various types of experimental asystolia and an effort has been made to elucidate the mechanism involved in this adrenergic response.

As working hypotheses the following possible mechanisms were considered:
1. If the supply of oxygen or oxidizable substrates is rate-limiting for oxidative phosphorylation, the calorogenic effect of adrenaline might be secondary to either an increased coronary flow or increased mobilization of endogenous substrates (lipolytic and glycogenolytic acti

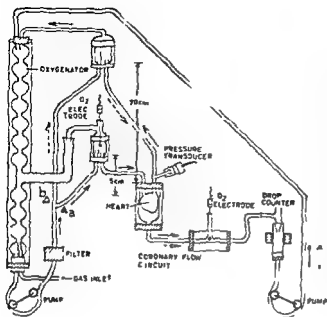


Fig 1 Perfusion arrangement. The perfusion method previously described (Øye, 1965) was modified to allow continuous recording of coronary flow and pO_2 . The coronary effluent is ejected through the open pulmonary artery into the closed chamber in which the heart is mounted and flows through a rigid tube (surrounded by a water jacket) into which the oxygen microelectrode is closely fitted through a side tube. Finally the coronary effluent flows through a photo-electric drop counter from which it is pumped back to the top of the oxygenator. Arrows show flow direction during anterograde perfusion (tube to upper reservoir clamped at Δ) and broken arrows show flow direction during retrograde perfusion (tube to lower reservoir clamped at Δ).

2. If the oxidative phosphorylation is limited by the rate of energy utilization the calorigenic effect of adrenaline might be secondary to either an increased utilization of ATP, or to an 'uncoupling' of the oxidative phosphorylation process.

Experiments were designed to examine the main determinants for cardiac oxygen consumption during asystolia, and possible target systems through which adrenaline might influence these determinants are discussed.

Methods

Rats of both sexes, about 300 g, local strain, originally Wistar, were used. The hearts were excised under ether anaesthesia and perfused by the method developed by Morgan (1961) as previously described (Øye 1965). This perfusion technique involves cannulation of the left atrium as well as the aorta. The left atrium is connected to an overflow reservoir 5 cm above the level of the heart and the aortic cannula is connected to a similar reservoir 75 cm above the heart. When the tube leading to the left atrium is open, perfusate will enter the heart through the left atrium and is pumped by the heart to the upper reservoir, thus allowing the heart to perform a physiological type of work *in vivo* (anterograde perfusion). When the tube leading to the left atrium is closed the perfusate will flow retrogradely through the aorta and coronary vascular bed as in a classical Langendorff type of perfusion (retrograde perfusion). In both cases the coronary effluent is expelled through the right ventricle into the closed chamber where the heart is mounted. In order to measure cardiac oxygen usage the perfusion circuit was modified as shown in Fig. 1, allowing continuous recording of coronary pO_2 with a Clark oxygen microelectrode and a Beckman Physiological Gas Analyzer Model 160. Coronary flow was measured with a Palmer phototransistor drop counter. Inflow pO_2 was measured by mounting the oxygen electrode in the reservoir connected to the left atrium. Oxygen consumption was calculated from the arterio-venous pO_2 difference and the oxygen solubility constant (L. ml/bm³ at 19.7). The perfusate was a modified Krebs Ringer bicarbonate buffer (Øye 1965), gassed continuously with 95% oxygen and 5% carbon dioxide. The buffer contained 1 per cent albumin (bovine fraction V, Sigma) and 10 mM glucose. Other additions are stated under results. The total volume of perfusate was 40 ml and the temperature was kept constant at 32° C.

Lactate was measured enzymatically with lactate dehydrogenase and NAD according to Hill (1963). Glycerol was measured according to Burton (1957).

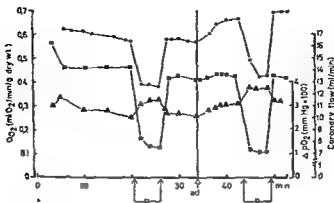


Fig 2 Oxygen consumption (QO_2 —●—) coronary flow (—■—) and arterio-venous pO_2 difference (ΔpO_2 —▲—) during anterograde and retrograde perfusion

After 20 min of anterograde perfusion flow direction was changed to retrograde perfusion (no pump work done by the heart) for five min (R). At 30 min $3\mu g$ adrenaline was added. As shown by the figure the increase in QO_2 in the subsequent periods of anterograde and retrograde (R) perfusion is mainly due to an increase in ΔpO_2 .

Adrenaline bitartrate (Rhône Poulenc) was dissolved in 0.9 per cent sodium chloride containing 1 mM EDTA and stored frozen. The adrenergic receptor blocking agents used were phenolamine (Regitin® Ciba), propranolol (Inderal® ICI) and dichlorophenylisopropylaminoethanol (DCI Aldrich Chemical Co Inc). The procainamide solution used was the commercial preparation Pronestyl®, Squibb.

Results

Oxygen consumption in the working and arrested heart

The average oxygen consumption of the isolated, working rat heart at 32°C, under the present experimental conditions, was 0.56 ml/min per g dry weight or about 13 ml/min per 100 g wet weight, (the wet weight/dry weight ratio in hearts perfused for 30 min being about 4.5). Fig 2 illustrates the typical change in oxygen consumption (QO_2), coronary flow and arterio-venous pO_2 differences (ΔpO_2) when the flow direction of the perfusate was changed from anterograde perfusion to retrograde perfusion, before and after the addition of adrenaline. During the period of anterograde perfusion the left ventricle contracted against a diastolic pressure of 75 cm perfusate. During the period of retrograde perfusion the perfusate was flushed through the aorta and the coronary vascular bed. The left ventricle was not filled during the diastole, and no external work (pumping) was done, as shown by no increase in the recorded aortic pressure during systole. Usually no change in rate or rhythm occurred when the flow direction was shifted from anterograde to retrograde. The mean value for oxygen consumption during retrograde perfusion was 70 per cent of the oxygen consumption during work.

A further decrease in oxygen consumption occurred when the hearts were made asystolic by increasing the extracellular potassium concentration by adding sodium citrate or by the use of procainamide. The oxygen consumption during asystolia

TABLE 1 Oxygen consumption in perfused hearts during work and in the non working state. Ten hearts were perfused by antegrade perfusion as described under methods for 15–20 min in order to obtain steady values for coronary flow and pO_2 . Aystolia was induced by exchanging 10 ml of the perfusate with 10 ml of a similar medium in which potassium had been substituted for sodium giving a final concentration of 34 me/l of potassium (group I) or by the addition of 2 ml 1 M sodium citrate to give a final concentration of 48 M (group II) or by adding 0.2 g procainamide to make 15 mg/ml (group III). During aystolia the hearts were perfused retrogradely through the aorta. In group IV retrograde perfusion was established by clamping the tube leading to the left atrium.

Experimental group	Conditions during experimental period	Oxygen consumption	
		Control period	Experimental period
		O ₂ in ml per min per g dry weight	
I	Arrested with potassium (17)	0.50 ± 0.04	0.16 ± 0.02
II	Arrested with pronestyl [®] 8	0.50 ± 0.04	0.27 ± 0.04
III	Arrested with citrate 8	0.50 ± 0.05	0.38 ± 0.02
IV	Reversion of flow to retrograde perfusion 4	0.58 ± 0.06	0.41 ± 0.02

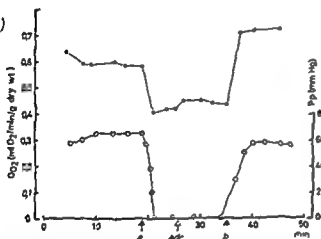


Fig. 3 Oxygen consumption and pulse pressure before and after the addition of sodium citrate final concentration 30 mM at 20 min. A frenaline 3 mg was added at 25 min. At 35 min 0.1 M CaCl_2 was infused until the pulse pressure reached normal levels. After a frenaline 2 mg increased in the aystolic as well as in the working state.

depended on the method used to arrest the heart. Table 1. In hearts arrested with citrate addition of CaCl_2 restored contractions and brought oxygen consumption to control levels. Fig. 3. In hearts arrested with potassium or procainamide contractions and oxygen consumption were restored on renewal of the perfusate.

The possibility that coronary flow *per se* is a determinant of QO_2 during aystolia was tested by adding adenosine which is known to reduce resistance to flow through the coronary vascular bed. Berne 1964. 1 mmole/l of this nucleoside increased flow rate in hearts arrested with potassium to an extent which resulted in a raised

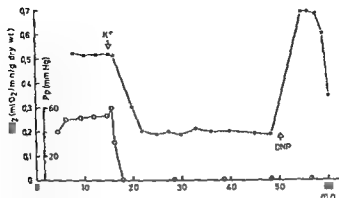


Fig 4 Effect of dinitrophenol

Oxygen consumption (—●—) and pulse pressure (---○---) in a heart arrested with high concentrations of potassium. At 16 min infusion of the potassium buffer was started. At 50 min dinitrophenol (final concentration 1 mM) was added to the perfusate.

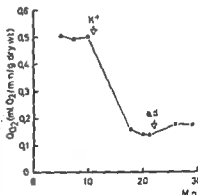


Fig 5 A typical experiment showing the effect of adrenaline on oxygen consumption during asystolia.

The heart was arrested at 15 min by increasing the potassium concentration as described under "methods". At 24 min 10 μ g adrenaline was added to the perfusate.

Q_{O_2} despite decreased arteriovenous pO_2 difference. However, when the flow rate was kept constant by mechanical regulation of the perfusion rate, adenosine was without effect on oxygen consumption. The nucleotides 5'-AMP and cyclic 3',5'-AMP had effects similar to adenosine.

Additional supply of substrates (5 mmols each of pyruvate, lactate and beta-hydroxybutyrate) was without significant effect on oxygen consumption of the arrested hearts. Since the albumin used contained a substantial amount of fatty acids, the effect of substrate addition to hearts perfused for some time in the complete absence of substrates was not tested.

The rapid adjustment of Q_{O_2} to changes in the physical activity indicates that energy utilization is a main determinant of Q_{O_2} in the isolated perfused heart. Restriction of oxygen consumption by the rate of oxidative phosphorylation persists also in the asystolic state as demonstrated by the dramatic effect of agents which uncouple oxidative phosphorylation. Fig 4 shows a typical experiment with a rela-

rat heart, it appears unlikely that the rapid calorogenic response observed in our experiments are secondary to the glycolytic or lipolytic effects. This does not exclude the possibility that an increased amount of free fatty acids contributes substantially to the calorogenic effects of adrenaline during longer periods of perfusion.

A role of coronary flow as a determinant of oxygen consumption during experimental asystolia was revealed by the use of adenosine and the related nucleotides 5'-AMP and 3',5'-AMP. However, the calorogenic effect of adrenaline did not depend on an increase in coronary flow and was found to be present even when adrenaline caused a marked decrease in flow.

If cardiac metabolism is interfered with, loss of the functional properties rapidly occurs (Bing 1965). The reversibility of the present preparation from the asystolic to the working state indicates that the essential metabolic processes were maintained during the period of asystolia and it is reasonable to assume that they were more or less subject to the same basic control mechanism as in the working heart. The effect of 2,4-dinitrophenol shows that the respiratory control through oxidative phosphorylation persisted. Under such conditions enhanced energy expenditure leads to increased oxygen consumption by increasing the amount of ADP and P_i available for oxidative phosphorylation. Adrenaline is known to stimulate biochemical reactions that use ATP in non working tissues. A well known example is the phosphorylation of phosphorylase b to form phosphorylase a (Krebs *et al.* 1964). In this process 4 moles of ATP are used to convert 2 moles of phosphorylase b to 1 mole of phosphorylase a, and 4 moles of ADP are formed. Phosphorylase a is rapidly dephosphorylated by a phosphatase to form phosphorylase b and P_i , the net result of the cycle being the formation of 4 ADP and 4 P_i . Glycogen synthetase (Rosell Perez and Larner, 1964) and possibly also lipase (Rizzick 1963) are subject to similar phosphorylation processes which appear to be stimulated by adrenaline. Adrenaline thus increases the energy expenditure not only in the contracting muscle but also in the asystolic heart. The inotropic and chronotropic effects in the beating heart as well as the initiation of contractions during experimental asystolia can be regarded as representing a direct or indirect stimulation of reactions which require energy. Increased ATP utilization appears to be a common denominator for the various expressions of adrenergic stimulation of cardiac muscle. This applies to the metabolic actions of adrenaline as well as to its effects on the physical activity of the muscle. It thus seems justified to explain at least part of the calorogenic effect of adrenaline during experimental asystolia as secondary to increased utilization of ATP by several separate reaction systems all of which contribute to the basal energy expenditure of the heart.

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Importance of Tissue Pressure for the Fluid Equilibrium between the Vascular and Interstitial Compartments in the Small Intestine

By

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Abstract

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The effect of changing the venous pressure during this procedure the rate of net fluid transfer upon a given venous pressure level could differ very much depending on whether this pressure level was reached from a lower or from a higher venous outflow pressure. The transcapillary forces responsible for these differences of net fluid transfer amounted to often more than 10 mm Hg. This means that some factor or factors involved in the Starling equilibrium must have changed by this amount. It is suggested that increasing tissue pressure is the most important counterforce during outward filtration, caused by a sudden rise of the venous outflow pressure. Evidently the small intestine is quite well protected against edema in spite of a very big transcapillary filtration capacity.

The relationship between intestinal blood flow resistance and capillary filtration coefficient (C_fC) was described in a previous paper (Jolkow, Lundgren and Wallentin 1963). The small intestine was shown to have a big capacity for fluid transport over the capillary membrane. C_fC, calculated for the mucosa portion, appeared to be in the magnitude of 1 ml/min/mm Hg/100 g tissue; at maximal vasodilatation, a figure in the range of the glomerular filtration in the human kidneys. The maximal blood flow in the mucosa was deduced to be about 500 ml/min/100 g tissue, i.e. some 10 times that of a skeletal muscle.

The big capacity for capillary transfer in the intestine seems to be well suited for the specialized functions of the mucosa as regards secretion and absorption. It implies, however, a potential danger that even slight increases in mean capillary pressure might rapidly cause an extensive intestinal edema.

Fortunately, the control of the mean capillary pressure in the intestine seems to be extremely good

1 Differences in hydrostatic pressure in intestinal capillaries, depending on the position of the intestinal loops in relation to the heart, are counterbalanced by similar hydrostatic pressure differences in the surrounding abdominal contents, opposing the tendency for pooling of blood (Rushmer 1946) and also fluid losses owing to increased filtration

2 The autoregulation of blood flow for different arterial pressure levels is pronounced (Johnson 1960, 1964) and seems to be more adapted for regulation of capillary pressure than for nutritional blood flow (Johnson 1964)

3 Changes in vasoconstrictor tone, whether these changes are induced reflexly (Öberg 1964), or by stimulation of the sympathetic vasoconstrictor fibres (Folkow *et al* 1964 a, b), do not modify the transcapillary fluid balance in the intestine, indicating an unchanged mean capillary pressure. In the skeletal muscle on the other hand, increased vasoconstrictor tone lowers the mean capillary pressure, which causes an often extensive absorption of fluid (Mellander 1960, Öberg 1964)

If, on the other hand, a primary rise in venous pressure occurs, active vascular adjustments have far less chance to counteract the influence of the venous pressure rise on the capillary pressure level. There must be many situations in which venous pressure really becomes raised, as in portal hypertension due to liver cirrhosis, right heart insufficiency, subclinical and manifest strangulation ileus, etc. Of course, it must be remembered that several other factors, besides the capillary pressure, determine the transcapillary fluid balance. The well known Starling equilibrium implies that the mean hydrostatic capillary pressure minus tissue pressure equals plasma colloid osmotic pressure minus tissue colloid osmotic pressure when no net transcapillary transport occurs. Therefore if mean capillary pressure is raised, it will cause an outward filtration, which theoretically produces a lowering of the colloid osmotic pressure in the interstitial fluid, an increase in the plasma colloid osmotic pressure and an increase in tissue pressure, all events which will counteract further filtration.

It was for such reasons considered to be of interest to know what happens in the transcapillary filtration/absorption exchange during prolonged increases in venous outflow pressure, and to discuss the relative importance of the different factors in the Starling equilibrium.

Methods

The method is similar to that of Johnson and Wallentin (1963) and this study was carried out in cats weighing 2–4 kg (mentioned as required) of the small intestine. After heparinization the superior mesenteric vein draining the intestinal segment and its lymph glands was cannulated and the venous outflow passed an optical drop recorder–ordinate writer unit after which it was returned to the animal. The intestine was autoperfused via a polyethylene tube from one carotid artery to the superior mesenteric artery. The intestinal pedicle was divided so that the preparation was totally denervated. Arterial blood pressure was measured from one femoral artery. The venous

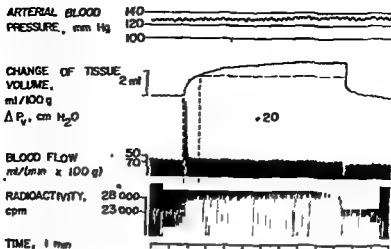


Fig. 1 Cat 2 kg. Effects of a prolonged period of raised venous outflow pressure on tissue volume and on the regional blood volume (the curve of radioactivity).

Note that the volume increase representing the outward filtration, is decreasing gradually, reaching a new isovolumetric state after about 8 min.

outflow pressure measured with a saline manometer could be set at any desired level by adjusting the height of the tube end which drained the drop recorder. The intestine, now only connected to

The temperature of the animals was maintained by an electric heating pad and an infrared lamp. Great care was taken to maintain a constant temperature for the intestinal preparation in the pletysmograph.

Intestinal lymph was collected in 3 cats. A polyethylene catheter was inserted in one of the biggest lymph vessels in the pedicle and the lymph was collected for periods of 15 or 30 min at different venous outflow pressures.

As an increase in the regional blood volume an

20,000—30,000 cpm.

Results

To determine the capillary filtration coefficient (CFC) venous outflow pressure is raised for a relatively short period, 1 1/2—3 min. This produces an initial rapid increase in the intestinal volume, due to venous distension, followed by a slower phase of increase, due to outward filtration. The filtration slope is proportional to

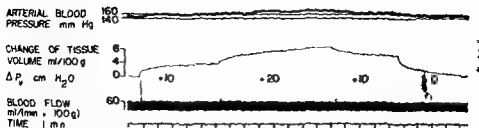
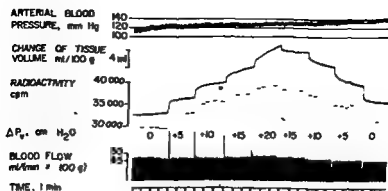


Fig 2 Cat 2.3 kg. The venous outflow pressure is raised stepwise by 10 cm water and thereafter lowered again. Note the tendency for the intestinal volume to level off towards an isovolumetric state both at raised and lowered venous pressure.

the rise in mean capillary pressure and the capillary surface area open to flow and hence for filtration. However, in the present investigation when the period of increased outflow pressure was prolonged, the filtration rate decreased gradually and often ceased more or less completely within 10–15 min. This typical response can be seen in Fig 1 where the outflow pressure was raised by 20 cm water. In this case the reactivity of the resistance vessels and precapillary sphincters was diminished by a constant intraarterial infusion of isopropyl noradrenaline (Pafadrin, AB Hässle) ca 0.3 µg/min. The changes in radioactivity emitted from the Cr^{51} labelled erythrocytes indicate that the blood content in the preparation increased when the venous pressure was raised, first very rapidly and thereafter at a slower rate during 40–50 sec, indicating an obvious delayed compliance of the capacitance vessels. During the rest of the period of raised venous pressure a fairly small and steady increase in radioactivity is seen. In case this increase also corresponds to increasing blood volume it amounts to only 15–20 per cent of the simultaneous increase in tissue volume. Another possibility is that it corresponds to small areas with very slow blood circulation, e.g. mesenteric fat or areas close to the ligated ends of the intestine. The record of tissue volume change, on the other hand, shows that the filtration is decreasing gradually during the whole period, reaching a new isovolumetric state after about 6–7 min. This means that a rise of 20 cm water in venous pressure, corresponding to about 12.5 mm Hg rise in mean capillary pressure (Folkow, Lundgren and Wallentin 1963) and a consequent filtration, was gradually balanced by some counterforce. Provided that no gradual shift in vascular tone changing the pre/postcapillary resistance ratio occurred, some of the other factors of the Starling equilibrium must have been changed. This tendency to level off gradually was seen both when raising and lowering the venous pressure as illustrated in Fig 2. Here the outflow pressure was raised in steps of 10 cm water and then lowered again. Thus, normally a new isovolumetric state was ultimately reached even when stepwise increases in capillary pressure were performed. Sometimes however a small volume increment, about 5–10 per cent of the initial outward filtration, continued even after a prolonged period of capillary pressure, possibly reflecting filtration from serosal and mucosal vessels, the amount of which is dependent on the degree of hyperemia in



volume

Observe that in case of no counterforces against the filtration each pressure level should cause the same amount of outward filtration independently whether this pressure level was preceded by a higher or lower venous outflow pressure

a filtration loss can, of course, not be counterbalanced by *e.g.* increases in tissue pressure or by dilution of interstitial proteins. It should perhaps be mentioned here that cats with infections, especially intestinal ones, often developed distended, edematous intestines, and in these cases rapid and fairly stable volume increases occurred whenever venous outflow pressure was kept increased. Cats, with evidence of such a pathophysiological disturbance of the transcapillary fluid exchange, could therefore not be utilized for the present purpose, where in the first hand the normal reactions were to be studied.

Fig. 3 shows what happens when the outflow pressure is increased in equal steps lasting about 3 min and then lowered successively again. It is obvious from the figure that any level of increased venous pressure causes filtration during the way up, but an absorption of fluid during the way down. The radioactivity levels show that the blood content after the period of delayed compliance (40–50 sec) is fairly constant at most pressure levels. Where obvious shifts in blood volume occur, *e.g.* at 20 cm water, these changes are corrected for during the evaluation of the filtration slopes. These corrections are, however, quite small. The slopes at each venous pressure level, corresponding to filtration or absorption are plotted in Fig. 4 and constitute, when connected, a characteristic loop, which was obtained every time the venous pressure was raised stepwise and thereafter lowered again, as in Fig. 3. The increase of filtration between each step from +5 to +20 cm water is fairly constant, indicating that also CFC is nearly constant during this period. The intersections with the abscissa denote zero slope, i.e. when no filtration or absorption occurs, and show that isovolumetric state can be reached at different levels of venous outflow pressure, in this case more than 15 cm H₂O apart, during this run of stepwise raising and lowering the venous pressure. The vertical distance between

Fig 4 The slopes of filtration or absorption from Fig 3 are plotted and connected to make a loop. On each level of venous outflow pressure two values are seen, one representing the outward filtration when the venous pressure is raised the other representing the absorption when the pressure is thereafter lowered. Zero slope is the isovolumetric state reached at different levels more than 15 cm apart. The vertical distance between the two parts of the loop represents the difference in net fluid transfer at each pressure level. At +10 cm water this difference amounts to 0.85 ml/(min \times 100 g). When divided by the simultaneous CFC 0.064 ml/(min \times mm Hg \times 100 g) the corresponding change in the Starling equilibrium in this case 13 mm Hg is known.

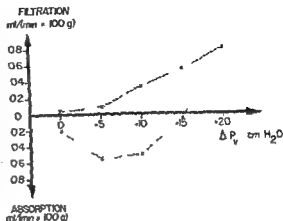
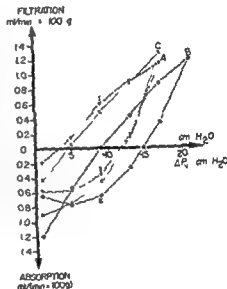


Fig 5 Each loop A, B and C, are plotted similar to the loop in Fig 4. Loop A represents a control run of stepwise changing the venous outflow pressure as in Fig 3. Thereafter a prolonged outward filtration was caused by raising the venous pressure by 13 cm water during 7 min (Not shown in the figure). Loop B, representing a run just after the venous pressure was lowered again, shows that additional filling of the tissue spaces with filtrate displaces the loop to the right. 20 min later when the fluid filtered during the long filtration period was absorbed again, a new run (loop C) was almost identical with the control.



the two parts of the loop expresses the difference in the rate of net fluid transfer at the same level of venous outflow pressure. When this difference is divided by the CFC obtained at the same level, the corresponding change in the Starling equilibrium which could explain this difference is known. In Fig 4 the maximal difference in net fluid transfer is obtained at +10 cm water and amounts to 0.85 ml/(min \times 100 g tissue). CFC is calculated to 0.064 ml/min \times mm Hg \times 100 g tissue. This gives in this particular experiment a figure of about 13 mm Hg pressure difference in the Starling equilibrium. The mean value of the maximal differences for 44 loops from 11 cats was 9.1 ± 2.33 SD mm Hg. This must mean that either the tissue pressure has increased or/and the interstitial colloid osmotic pressure dro-

static capillary pressure has decreased by a corresponding amount. Any of these factors alone, or in combination, can, of course, be responsible here. There are no reasons to assume that the colloid osmotic pressure of the blood has changed to any significant extent (see below).

Intraarterial infusions of papaverine HCl, in doses sufficiently high to eliminate completely the tone and reactivity of the resistance vessels (1–3.5 mg/min) did not change these loops or diminish significantly the maximal difference mentioned above. This proves that the mentioned events are not consequences of slow vascular adjustments causing capillary pressure changes, but reflect changes of either tissue pressure or/and interstitial colloid osmotic pressure.

In Fig. 5 each loop (A, B and C) represents a run of stepwise raising and lowering venous pressure as in Fig. 3 and 4. After a control run (loop A), the venous pressure was raised with 13 cm water, producing an outward filtration, which after 7 min had decreased to about 15 per cent of the initial value. (This long filtration period is not shown in the figure). After lowering the venous pressure again, new runs were performed, first immediately (loop B) and then 20 min later, when the fluid filtered just before loop B had been absorbed again (loop C). Obviously, the vertical distance between the points I and II of loop A as compared with point I of loop A and point III of loop B now indicates that the difference between two different slopes at the same outflow pressure can be augmented further, in this case from 8 to 10 mm Hg, by additional filling of the interstitial tissue spaces with capillary filtrate. The displacement of the isovolumetric state to higher venous pressures (i.e. the intersections with the abscissa) is of course a reflection of the same phenomenon.

The mean intestinal lymph flow measured in 3 cats was 0.55 ml/(30 min \times 100 g intestine), increasing to 1.10 ml/30 min during an increase in venous outflow pressure of 20 cm water. The corresponding mean outward filtration was 7.5 ml/(30 min \times 100 g). It is important to stress that allowing for the drainage of lymph did not change the configuration of the volume recordings as seen in Fig. 1.

Discussion

The experiments show that the intestine has a great capacity to neutralize considerable changes in capillary pressure. E.g. in Fig. 1 a new isovolumetric state was reached 6–7 min after the venous outflow pressure was raised by 20 cm water. Johnson and Hanson (1963) came to a similar result with their isogravimetric technique. They plotted the weight changes after elevating the venous pressure on a semilogarithmic scale and found two distinct phases, a rapid one which they interpreted as corresponding to the venous distension and a slow one corresponding to the capillary filtration. The rapid phase had a time constant of 10.9 sec, which means that after 3 time constants, ca. 33 sec, 95 per cent of the venous filling was completed and after this time the volume increase consisted mainly of filtration. This agrees very well with the present results where the same phenomenon is seen during the first 40–50 sec on the isotope record of the blood volume. Johnson and Hanson

did not discuss in their paper why the filtration also decreased exponentially, but in a later paper (Johnson 1965) it was explained as due to dilution of the interstitial proteins (see below). As, however, several different factors may contribute to this new Starling equilibrium at a higher capillary pressure level, such factors will now be discussed more at length.

The venous arteriolar response When the portal venous pressure was elevated in dogs, the blood flow decreased relatively more than the perfusion pressure. Johnson (1959, 1960) showed that this was caused by a myogenic response, predominantly of the precapillary resistance vessels, which constricted when they were stretched by the raised pressure. This by now well known response of many different types of smooth muscle to stretch was originally suggested for vessels by Bayliss (1902). Two different effects of this venous arteriolar response are possible. 1 The precapillary constriction is effective enough to again lower the raised capillary pressure, caused by the primary rise in venous pressure (Johnson 1965). 2 The precapillary sphincters, which are known to be especially sensitive for pressure changes (Mellander, Öberg and Odelram 1964), constrict and therefore reduce the perfused capillary surface area, as determined by CFC. Such a reaction will reduce the extent of outward filtration even in case the capillary pressure rise would remain the same. However, it will not abolish the outward filtration except for the case that all capillaries become closed and the direction of the net fluid transfer can never be reversed by any changes in CFC. In general, it seems unlikely that a myogenic response to a sudden stretch, or unloading, would have a time course so prolonged as to give the smooth changes in filtration rate seen in Fig. 1 and 2. However, even if these myogenic responses are very important *per se* for counteracting primary changes in capillary pressure, they can not be significantly involved in these experiments for following reasons.

Isopropyl noradrenaline was always infused at such concentrations as to depress markedly, or even eliminate entirely, the reactivity of the resistance vessels and venous arteriolar responses were therefore not seen in the present experiments. Also papaverine HCl in doses which are considered to abolish all vascular reactivity (Mellander and Lundgren 1966) did not decrease the maximal difference in net fluid transfer at the same outflow pressure. Moreover, in case there was some reactivity left after administration of these drugs it would then be expected that these 'active' adjustments would be of largely the same magnitude at equal rises of capillary pressure, whether these rises occurred during the phase of filtration or the phase of absorption of the loop. It follows that such active adjustments can not explain the considerable difference in direction of the slopes.

Plasma colloid osmotic pressure A 3 kg cat has a plasma volume of about 150 ml (Hamlin and Gregersen 1939). The intestinal section used during the experiments weighed about 40 g and accumulated a maximum of 2–3 ml filtrate during the phase of outward filtration when a run of stepwise increased venous pressure was performed. Even in case the filtered fluid was totally protein free the plasma proteins would become only slightly concentrated by this fluid loss, corresponding to

an increase in the plasma colloid osmotic pressure by less than 0.5 mm Hg, a figure without significance for the present problem. Furthermore, any increase in concentration of the plasma colloids will cause an inward absorption in other vascular beds, which will dilute the proteins again, thereby opposing the tendency to change the plasma colloid osmotic pressure.

Tissue colloid osmotic pressure Dilution of the interstitial proteins lowers the interstitial colloid osmotic pressure and counteracts an elevated mean hydrostatic pressure in the capillaries. Johnson (1965) reported that the dog intestine became isogravimetric at any venous outflow pressure between 0–18 mm Hg, and suggested that this was essentially due only to the dilution of the interstitial proteins, since tissue pressure, measured by a needle technique, was not affected even by large changes in venous pressure. This will be commented on below.

The present experiments have shown that differences in net fluid transport at the same venous outflow pressure correspond to a maximal change of often more than 10 mm Hg (maximally 14.8 mm Hg) in some factor, or factors, of the Starling equilibrium. However, the effect of lowering the tissue colloid osmotic pressure must be limited by the initial value before the dilution. This value must be very variable during different functional situations in the intestine, but a mean value of about 5 mm Hg was considered reasonable by Landis and Pappenheimer (1963). Even if the tissue colloid osmotic pressure in the intestine was considerably higher, theoretical difficulties remain. Assume for a moment an interstitial colloid osmotic pressure as high as 15 mm Hg, which corresponds to a concentration of about 5 g of protein/100 ml. To lower this pressure to 5 mm Hg the proteins must be diluted to 1.7 g/100 ml (Landis and Pappenheimer 1963), a dilution of the interstitial fluid approximately 2.5 times. In the present experiments the difference in filtered fluid on that level of outflow pressure, which had the biggest difference in net fluid transfer, was very seldom more than 1–2.5 ml per 40 g intestine. If a decrease of interstitial colloid osmotic pressure was the only factor involved, it would mean that only 0.67–1.67 ml per 40 g intestine (= 1.7–4.2 per cent) is interstitial fluid, an improbably low figure.

Moreover, it is very difficult to assume a mean value of proteins in the intestinal fluid as high as 4–5 g/100 ml. Morris (1956) collected intestinal lymph from cats and found a protein concentration of 4.19 ± 0.29 g. As in other tissues the concentration of proteins in the lymph ought to be considerably higher than that of the interstitial fluid. Landis and Pappenheimer (1963) calculated that the continuously produced capillary filtrate in a resting animal was at an average at least 5–10 times greater than the average lymph flow. For many reasons therefore, it seems impossible that dilution of the interstitial proteins could be responsible for more than 4–6 mm Hg of the deduced maximal difference in the Starling equilibrium. This large difference up to about 14–15 mm Hg was, as mentioned, deduced from loops, as illustrated in e.g. Fig. 4, where both the amount of filtered fluid and CFC are known.

The tissue pressure As mentioned, Johnson (1964) did not find any increase in tissue pressure with the needle technique. By a similar method Meyer, Nordheim and Ballin

(1962) found that the

did not increase du

but increased significantly during the edema of endotoxin shock. However, the methods to measure tissue pressure by needle techniques have been criticized for many reasons perhaps the most important one is the fact that even the finest needle is several hundred times larger than the tissue spaces, where the pressure should be recorded (McMaster 1946, Guyton 1953). When the connective tissue is disrupted by heavy edema then the spaces are probably more adapted to the recording needles. On the other hand it is stated repeatedly, that in many tissues outward filtration is counteracted by an increasing tissue pressure (Landis and Gibbon 1933, human forearm, McMaster 1946, mouse skin, Kjellmer 1963, cat skeletal muscle). Theoretically, an increasing tissue pressure seems to be well adapted to the purpose of protection against edema. The smaller the distance between the

the inter

when the quantities other corrections will appear (McMaster 1941, 1946). Perhaps such a disruption of tissue spaces can explain why, in infectious intestines, increased venous pressure causes outward filtration during very long time. Tissue pressure can reach high values (McMaster (1946, mouse skin) and Landis and Gibbon (1933, human forearm) found tissue pressures up to about 25 mm Hg with prolonged filtration. When elevation of venous outflow pressure with 20–30 cm H_2O can be counteracted so that the outward filtration ceases, it seems very unlikely that this can be explained without considerable contribution of a raised tissue pressure.

In the present investigation the lymphatics were ligated in most cases (as in Johnson's experiments) and it is technically difficult to record lymph flow and intestinal volume simultaneously. It may then be questioned whether the assumed rise in tissue pressure is simply an artifact caused by the accumulation of lymph fluid. It is a well known fact that raised venous pressure will increase the rate of lymph production. However, there is usually a considerable delay before the rate of lymph flow increases to an extent that equals the rate of outward filtration (Morris 1956). Morris found an intestinal lymph flow in resting cat of about 4 ml per hr. After raising the portal pressure with 12 cm water it increased to 6 ml per hr but needed 60 min to reach this value. During the first 15 min period it amounted only to about 4 ml per hr, i.e. an increase with 0.5 per hr for a rise of 12 cm water = 0.008 ml per min. Assuming 100 g intestine and a mean CFC of 0.15 a rise of 12 cm water in outflow pressure, corresponding to 7 mm Hg increase in mean capillary pressure, should cause an outward filtration amounting to 0.15 \times 7.5 mm Hg = 1.13 ml per min if no counteracting factors become secondarily involved.

Since Morris' data were very extensive lymph was collected in only 3 experiments in the present investigation where simultaneously tissue volume was recorded. The mean rise in lymph flow was 0.33 ml per 30 min for 20 cm elevation in venous pressure, a figure quite comparable with those given by Morris. There is so great discrepancy between the calculated outward filtration in case no counter

are involved, and the corresponding measured increase in lymph flow, that ligating the lymph vessels can not be an important cause for the increase in tissue pressure deduced within periods of 10–15 min, when venous outflow pressure was raised

The present investigation has shown that the intestine is fairly effectively protected against edema during increases in venous outflow pressure, in spite of a very big capacity for net fluid transfer across the capillary wall. Differences in the Starling equilibrium up to about 14–15 mm Hg was deduced by the method of stepwise raising and lowering the venous pressure. It seems impossible to explain this difference without assuming an increasing tissue pressure when fluid is filtered into the tissue spaces. In the living animal, however, more mechanisms than tissue pressure and tissue colloid osmotic pressure, e.g. the venous arteriolar response, certainly play an important role in decreasing the potential risk of having the big capillary transfer capacity necessary as it is for the primary functions of the small intestine.

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¹ venous pressure

² 311–318

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Effect of Finnish Bath (Sauna) on the Urinary Excretion of Noradrenaline, Adrenaline and 3-Methoxy-4-Hydroxy-Mandelic Acid

By

M HUIKKO, P JOUPPIILA and N T KÄRKI

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Abstract

HUIKKO, M, P JOUPPIILA and N T KÄRKI *Effect of Finnish bath (sauna) on the urinary excretion of noradrenaline, adrenaline and 3-methoxy-4-hydroxy-mandelic acid* Acta physiol scand 1966 68 316—321

In the present work the urinary excretion of noradrenaline, adrenaline and 3-methoxy-4-hydroxy-

Many authors have shown that urinary excretion of noradrenaline increases as a result of physical effort (Luler and Hellner 1952, Kärki 1956), but also in situations of emotional tension, anger or aggressions without physical exertion (Elmadjian *et al* 1958, Levi 1961). Adrenaline excretion may also rise in these situations, though it is specifically increased by anxiety, unpleasant emotions and stress from work (Luler *et al* 1959, Pekkarinen *et al* 1961, Levi 1961).

On the other hand a pleasing situation, such as watching a scenic film, has been found to reduce significantly the excretion of adrenaline and noradrenaline (Levi 1963).

The Finnish sauna offers a special field for the study of catecholamine metabolism. Almost every Finn takes a sauna once a week. The majority find it emotionally a pleasant, relaxing experience, but it produces major changes in the circulatory system. The period spent in the sauna usually ranges from 30 to 60 min. According to Eisalo (1956), body temperature, recorded rectally, increases by 1—1.5° C due to the heat of the surroundings. Cutaneous blood vessels are dilated, and peripheral resistance falls significantly. Cardiac output in healthy subjects increases by 73 per

TABLE I Excretion of noradrenaline, adrenaline and 3-methoxy-4-hydroxy-mandelic acid during a Finnish bath (sauna)

Group	Subject	Noradrenaline excretion ng/min		Adrenaline excretion ng/min		3-methoxy-4-hydroxymandelic acid excretion μ g/min		Pulse beats per min	
		Control	Sauna	Control	Sauna	Control	Sauna	Control	Sauna
I (Controls after sauna)	1	29.6	66.5	7.1	9.5	6.0	7.4	82	124
	2	9.1	71.7	3.1	12.1	3.9	3.4	52	124
	3	13.3	81.9	6.2	14.1	4.2	3.9	75	122
	4	8.1	35.8	13.0	6.9	3.0	4.3	76	120
	5	24.6	66.5	6.1	6.1	6.3	6.0	90	130
	6	36.1	47.8	11.3	11.7	3.7	4.4	72	120
	7	32.0	42.0	3.0	3.0	4.4	4.8	—	—
	8	66.0	54.0	6.0	10.5	4.7	3.6	—	—
	9	51.0	51.3	3.7	5.7	6.2	4.2	62	86
II (Controls 24 hrs later)	10	40.3	33.0	8.4	21.0	3.8	5.3	110	83
	11	40.6	26.9	6.6	17.1	3.6	4.4	72	134
	12	23.4	48.0	7.6	13.0	3.2	6.9	52	140
	13	36.0	25.8	4.8	5.8	—	—	74	115
	14	39.0	39.6	9.5	6.9	—	—	62	82
	15	33.1	58.2	11.6	11.8	—	—	96	140
	16	18.0	28.2	5.4	3.8	—	—	70	96
	17	29.2	38.5	6.8	5.5	1.7	3.2	—	—
	18	19.5	23.1	5.5	6.4	3.8	3.2	—	—
Mean and S.E.M.		30.6 \pm 3.4	46.6 \pm 4.1	7.0 \pm 0.7	9.6 \pm 1.1	4.2 \pm 0.3	4.6 \pm 0.4	72.5 \pm 3.4	115 \pm 5.3

cent, and pulse rate by average of 61 per cent. Systolic and diastolic blood pressure remain practically unchanged.

In the present work the changes in the urinary excretion of noradrenaline, adrenaline and their major metabolite, 3-methoxy-4-hydroxy-mandelic acid due to the sauna bath has been studied.

Material and Methods

The experiment comprised 18 healthy male subjects, mean age 24 years, all of whom habitually took the sauna, where for this study they stayed 20–40 min. at a temperature of 70–80 °C and relative humidity of 20–40 per cent.

Before starting the bath the resting pulse rate, blood pressure and sublingual temperature of 14 test subjects was recorded. These recordings were repeated in the sauna after 20 min. immediately on leaving the sauna, and 20 min. later.

After the sauna bath the 14 test subjects emptied their bladders. Urine passed during the 2–3 hrs. re collected during the 2–3 hrs. other 10 subjects during a

corresponding to that of the sauna 24 hrs later.

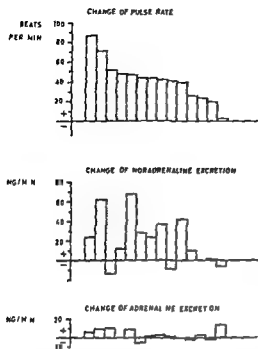


Fig 1 Changes in the pulse rate noradrenaline and adrenaline excretion in 14 test subjects during a Finnish bath (Sauna)

The pH of the collected urine sample was adjusted with 6 N hydrochloric acid to about 3 and the specimens were kept in the refrigerator until the determinations were performed. At this pH and temperature the catecholamine content remains constant over long periods (Karki 1956, Euler and Lishajko 1961).

The method for differential estimation of noradrenaline and adrenaline described by Crout (1961) was used. This method consists of adsorption of catecholamines in alumina at pH 8.4, elution with 0.2 N acetic acid, oxidation with iodine at pH 6.5 and 3.5 into aminochromes and

3-methoxy-4-hydroxy-mandelic acid was determined according to Pisano *et al.* (1962) by extracting the metabolite from the urine, oxidating it with periodate into vanillin and measuring the vanillin content with spectrophotometer at 360 m μ . A standard of 10 μ g 3-methoxy-4-hydroxy-mandelic acid was carried through the entire procedure in every series.

Creatinine was determined from the urine of 6 subjects using the alkaline picrate method (Reiner 1953).

Results

A Excretion of noradrenaline, adrenaline and 3-methoxy-4-hydroxy-mandelic acid into the urine

The mean value of noradrenaline excretion for control group I (after the sauna) was 27.3 ± 7.2 ng/min and for control group II (at the same hour 24 hrs later) 33.2 ± 3 ng/min. The difference between the readings for control groups I and II was not significant and therefore the mean of all control readings was used. Average excretion of noradrenaline into urine increased during the sauna, from the mean control value of 30.6 ± 3.4 ng/min to 46.6 ± 4.1 ng/min (Table I). This change is statistically significant ($P < 0.02$). Adrenaline excretion rose from 7.0 ± 0.7 ng/min to

9.6 ng/min, the difference is significant ($P < 0.05$). The control value obtained for 3-methoxy-4-hydroxy mandelic acid was 4.2 ± 0.3 μ g/min and the sauna value 4.6 ± 0.4 ng/min. The difference here is not significant ($P < 0.3$).

B *Change in pulse rate*

The resting pulse rates of the subjects ranged from 52 to 96 beats per minute, the average was 72 beats per minute. During the sauna, all test subjects showed an increase in pulse rate, by an average of 43 beats per minute ($P < 0.001$).

The change in the excretion of catecholamines in the 14 different test subjects is shown in Fig. 1 in the order in which the pulse rate increased.

C *Blood pressure*

Changes in the blood pressure during the sauna were slight. Control value recorded in sitting position ranged from 110/75 to 155/100 mm Hg, and the mean value was 130.8 mm Hg. The systolic pressure rose in the sauna by an average of 10 mm Hg, while the diastolic remained unchanged.

D *Body temperature*

Body temperature, recorded sublingually, increased in the test subjects by $0.1-0.3^{\circ}\text{C}$.

E *Creatinine excretion and catecholamines*

The creatinine values of test subjects 1-6 averaged 55 mg per hr during the control period and 72.0 mg per hr in the sauna, there was thus a distinct increase. The increases in the catecholamine excretion per time unit and per creatinine excretion were approximately of the same order: the total catecholamine excretion per time unit increased as a result of sauna by 155 per cent and per mg of creatinine by 140 per cent.

Discussion

A comparison of the changes noted in circulation in this study, with Eisalo's (1956) findings, shows that he recorded an identical rise in pulse rate. He also showed that the systolic and diastolic blood pressure of a healthy subject remained, on the average unchanged in the sauna, while the present authors recorded a slight rise in systolic blood pressure. The recorded control values for catecholamine excretion correspond in magnitude to those mentioned by other authors. It was also found that the control values taken immediately after the sauna sample and those obtained on the following day at the same hour were not significantly different. This agrees with the findings of Harkki (1956) who stated that the excretion in the same person remains nearly constant at the same hours on different days.

Bearing in mind the circulatory changes which are shown to be produced by body heating, and

present results which show that the sympathetic tone is involved in the sauna seem logical. As a result of surrounding heat the temperature of the skin increases, and due to reflex release of vasoconstrictor tone the cutaneous blood flow increases (Roddie *et al.* 1956). Apparently the widening of the vascular bed in the skin is compensated for by vasoconstriction in other areas to maintain blood pressure homeostasis. The observation that blood flow through internal organs diminishes during body heating supports this view (Grayson 1951). The circulatory changes are transmitted by sympathetic nerves, this higher level of activity leads to an increased liberation of noradrenaline from nerve endings and an increased excretion in urine.

Although factors known to increase adrenaline release are slight or missing, the slight increase in adrenaline excretion indicates that also adrenal medulla is stimulated. The percentage of adrenaline from total catecholamines was practically the same in sauna samples as in controls.

The excretion of 3-methoxy-4-hydroxy-mandelic acid has been found to reflect short-term sympathetic activity less well than does the excretion of free noradrenaline and adrenaline (Euler 1964). This was also revealed by the present study in that the increase in the excretion of 3-methoxy-4-hydroxy-mandelic acid was distinctly smaller and was not statistically significant. This phenomenon is attributed to the relative slowness of 3-methoxy-4-hydroxy-mandelic acid excretion (Euler 1964).

Little is known of the part played by the kidneys in the excretion of catecholamines and their metabolites but more of the influence of the sauna on renal function. Karvonen (1954, 1955) has attributed reduced urine excretion in the sauna to an increased liberation of antidiuretic hormone. A sauna bath of normal duration reduces glomerular filtration slightly but plasma flow is affected more deeply (Haapanen 1958). Those changes are considered to be due to the constriction of efferent glomerular arteries and are similar to the changes produced by injection of vasopressor substances. Whether or not those slight changes in renal function in the sauna affect the excretion of catecholamines remains to be elucidated.

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The Effect of DOPA on the Spinal Cord

3. Depolarization Evoked in the Central Terminals of Ipsilateral Ia Afferents by Volleys in the Flexor Reflex Afferents

By

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Abstract

The effect of DOPA on the spinal cord 3 Depolarization evoked in the central terminals of ipsilateral Ia afferents by volleys in the flexor reflex afferents Acta physiol scand 1966 68 322—336

In unanaesthetized spinal cats injected with 1 DOPA volleys in the flexor reflex afferents (FRA) evoke a long latency long lasting dorsal root potential (DRP). Measurements of excitability of ipsilateral primary afferent terminals show a corresponding depolarization of Ia but not Ib or cutaneous afferent terminals. Volleys in the FRA have no effect on Ia afferent terminals in the normal acute spinal cat presumably because the neuronal pathway transmitting the effect from the

DOPA and is probably caused by a removal of depolarization

In the acute spinal cat Ia afferent terminals are depolarized by volleys in Ia and Ib primary afferents, especially from flexor muscles (Eccles, Magni and Willis 1962), but not by stimulation of the flexor reflex afferents (FRA). Volleys in the FRA normally produce some depolarization of Ib terminals (Eccles, Schmidt and Willis 1963a) but their main effect is that exerted on their own terminals here presynaptic inhibition acts as a negative feedback (Eccles, Kostyuk and Schmidt 1962, Eccles, Schmidt and Willis 1963b, Lund, Lundberg and Vyalicky 1965).

The depolarization of primary afferent terminals is profoundly changed after intravenous injections of L-3,4 dihydroxyphenylalanine (DOPA). The normal dorsal root potentials (DRP) evoked by volleys in the FRA are depressed but an additional DRP appears which has a longer latency and a much longer duration (Andén *et al*

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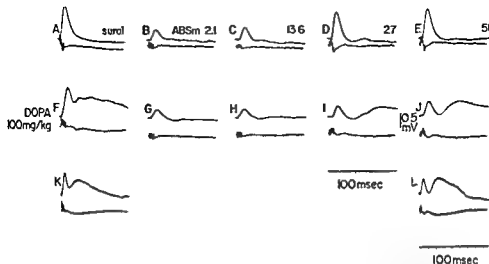


Fig. 1. The effect of DOPA on the DRPs evoked from the FR. The upper traces show the DRPs recorded from the most caudal dorsal rootlet in L5; the lower traces are recorded from the L7 dorsal rootlet.

DRPs

1966a) In the present paper it will be shown that the late DRP is caused by depolarization of the terminals of Ia afferents, therefore a previously undetected neuronal pathway exists in the spinal preparation, which permits depolarization of Ia terminals by volleys in the FRA. Some of the results have been briefly reported (Anden *et al* 1964).

Methods

The experiments were made on unanaesthetized spinal cats as described by Anden *et al* (1966a). Excitability measurements from primary afferent terminals were made as described by Wall (1958).

The following abbreviations are used: dorsal root potential (DRP), primary afferent depolarization (PAD), posterior biceps semitendinosus (PBSt), anterior biceps semimembranosus (ABSm), plantaris (Pl), gastrocnemius soleus (GS), posterior knee joint nerve (J), sural (Sur), flexor reflex afferents (FRA).

Results

1. The DRPs evoked from the FRA after DOPA

Trains of volleys were employed to evoke the DRPs shown in Fig. 1. The control records A-E, before DOPA was given, show the normal early DRPs with a duration of about 200 msec. DOPA reduced the early DRPs from cutaneous afferents (F) and from high threshold muscle afferents (I and L), but a late DRP appeared whose latency is about 100 msec in I and 150 msec in J. Records K and L, taken at slower speed, show the long duration of the late DRP.

low threshold cutaneous afferents did not evoke the late DRP, it appeared only when the stimulus strength was raised to about twice threshold. Volleys in group I muscle afferents never produced a late DRP and the muscle nerves usually had to be stimulated at a strength of 20–30 times threshold in order to evoke the late PAD. However, sometimes a late DRP could be evoked from group II afferents and occasionally from very low threshold group II afferents (Fig. 6).

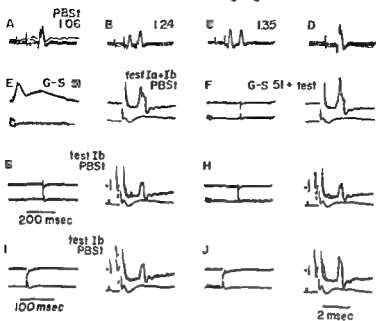
In a few of the experiments the late DRP failed to appear after DOPA had been given. It is of interest that in those experiments DOPA nevertheless effectively depressed the early DRP evoked from the FRA.

2. The recipient primary afferents

Excitability measurements were made to disclose which primary afferents were depolarized when the late DRP was evoked after DOPA had been injected. In the experiment of Fig. 2 the tip of the stimulating microelectrode was located in the G S motor nucleus and the antidromic Ia volleys recorded peripherally in the G S nerve. After DOPA a train of volleys in high threshold muscle afferents increased the excitability of the Ia terminals. The latency and time course of this increased excitability paralleled the late DRP evoked by the same train of volleys. There does not seem to be any difference in receptiveness between Ia terminals from extensor and flexor muscles, in the same experiment an equally large increase in excitability was found in the terminals of Ia afferents from the flexor PBSt (cf Fig. 9).

Fig. 3 illustrates an experiment in which the excitability of primary afferent terminals was tested with the tip of the stimulating electrode in the region of the intermediate nucleus of the spinal cord, where the extracellular focal potential evoked from Ia and Ib afferents was maximal. The group I volley from PBSt had good separation in Ia and Ib components (A, D) and the antidromic spike evoked from the intraspinal stimulating electrode and recorded in the PBSt nerve had a double configuration (E). The early component of the antidromic spike was a Ia volley, as shown by its abolition (record G and I) when a colliding maximal Ia volley was evoked from a second pair of electrodes on the PBSt nerve. The remaining antidromic volley was in Ib afferents as was proved by its disappearance with a maximal group I colliding volley. A train of volleys in high threshold muscle afferents which produced a late DRP (E) did not change the size of the Ib antidromic test volley (H and curve). Hence there was no increase in the excitability of the Ib terminals corresponding in time to the late DRP. The effect in J at a short conditioning testing interval, the time course of which can be seen in the early part of the curve, is the normal effect of group Ib muscle afferents (Eccles *et al.* 1963a) and shows that it was possible to obtain an increased excitability in Ib afferents in this experimental situation. Hence there is no evidence that a PAD in Ib afferents contributes to the late DRP after injection of DOPA. Fig. 3 F shows the increased excitability of Ia afferents when the test stimulation was applied in the region of the intermediate nucleus. It is likely that the increased excitability in this case was caused by

DOPA 100mg/kg



% of test

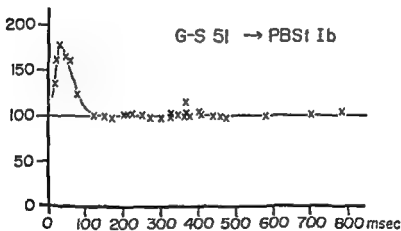
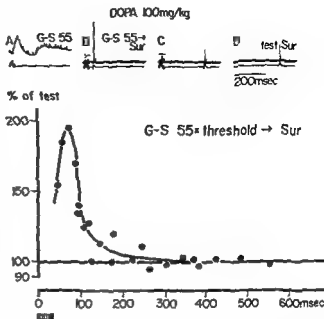


Fig 4 Measurement of the excitability of cutaneous afferent terminals when conditioned by volleys in the FRA after DOPA. Record A shows that a long latency DRP (upper trace) was evoked from high threshold muscle afferents after injection of DOPA (100 mg/kg), the lower traces are recorded from the L7 dorsal root entry zone. Records B and C show the antidromic volley recorded from the sural nerve evoked by local stimulation within the dorsal horn where the extracellular monosynaptic focal potential obtained by stimulation of the sural nerve was maximal. When conditioned by volleys in the high threshold afferents of the G-S nerve the test antidromic volley B increased at short conditioning test intervals (B), but not at longer intervals (C) corresponding to the late wave in the DRP, the time course of the increased excitability is plotted as in Fig 2.

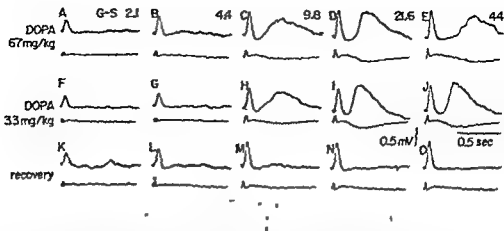


synaptic effects on the Ia terminals in this region. The PAD in these more dorsally located Ia terminals may very well give a major part of the late DRP.

The excitability of cutaneous afferent terminals was tested by stimulation through an electrode whose tip lay at the level in the dorsal horn where a large monosynaptic extracellular focal potential was evoked by stimulation of cutaneous afferents. In Fig 4 there is an initial increase in excitability caused by the early PAD from Ib afferents and from the FRA (cf Eccles *et al.* 1963b) but there was never any increased excitability in cutaneous afferents during the late DRP. No attempt was made to find out whether high threshold muscle afferents are depolarized from the FRA during the late DRP. However, this seems unlikely since cutaneous afferents and high threshold muscle afferents usually receive similar effects from the FRA. Hence our findings suggest that the late DRP evoked from the FRA after injection of DOPA is caused exclusively by a PAD in Ia afferents.

In some experiments stimulation of the FRA evoked a late wave in the DRP and a long latency discharge in the ventral roots without any DOPA being given. In addition there was always a depression of the usual short latency effects from the FRA. Measurements of the excitability of the terminals of Ia, Ib and cutaneous afferents showed that, at a time corresponding to the late DRP the excitability of the Ia terminals was increased by volleys in the FRA but not in group I afferents. No

afferents of the G-S nerve. The time course of the increased excitation of Ib afferents is plotted in Fig 2. Time calibration for the left hand records of E-H is shown in G and for the left hand records of I and J in I. All right hand records in E, J were taken at the faster speed in J.



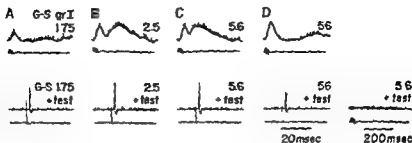
(67 mg/kg) and demonstrate the appearance of the late DRP with activation of the FRA (C) and the increase of its latency as stimulation strength was increased (D-E). This latter effect was not so marked (H-J) after an additional 33 mg DOPA/kg had been injected. Records K-O were taken 2 hrs later when the effects of the DOPA injections had worn off and no late wave could be evoked by activation of the FRA. Time scale applies to all records; voltage calibration to all DRPs.

increased excitability of Ib or cutaneous afferent terminals at this latency was found. It seems that under some conditions modifications of spinal reflexes may occur in the normal acute spinal preparation which usually only occur after injection of DOPA.

3 Inhibition of the late DRP

Fig. 5 shows that the latency of the late DRP is prolonged when the strength of stimulation is increased. After injection of 67 mg/kg of DOPA the magnitude of the late DRP increased somewhat when the stimulus strength was raised from 9.8 to 21.6 times threshold (D). With further increase of the stimulus strength (E) the most marked effect was a prolongation of the latency but there was also some decrease of the size of late DRP. F-J were taken after an additional dose of DOPA 33 mg/kg. The DRP evoked at 9.8 times threshold in H was almost the same as in C but with increase of stimulus strength in I and J the prolongation of the latency, although still present, was not so marked as in D and E. Furthermore there was no decrease of the late DRP in J when the stimulus strength was raised to 44 times threshold. Records K-O were taken 2 hrs later when the cat had recovered from the effect of DOPA. The early DRPs from the FRA had increased slightly and were not followed by late waves. The decrease in magnitude of the late wave with increasing strength of stimulation may be more marked than in Fig. 5. In the experiment of Fig. 6 the late DRP was evoked from low threshold group II afferents (B₁) and when group III afferents were also stimulated (D₁) the late wave was almost abolished. Correspondingly there was a removal of the increased excitability in the Ia afferent terminals with increased strength of stimulation (lower records). From these results it is inferred that stimulation of the FRA not only evokes the late DRPs but can also inhibit them. Another indication of this inhibition is given with prolonged repetitive stimulation

DOPA 100mg/kg



DOPA 67mg/kg



Fig 7 The effect of prolonging repetitive stimulation on the latency and amplitude of the late wave in the DRP evoked after DOPA. Upper traces show the DRPs, lower traces the potentials recorded from the dorsal root entry zone, evoked by repetitive stimulation of the G-S nerve at 45 times threshold after injection of DOPA (67 mg/kg). Note both the increase in latency of the late wave of the DRP and the decrease in amplitude. Same time scale for all records, the voltage scale applies to all DRPs.

of the FRA. Fig 7 shows that whatever the duration of the repetitive stimulation the late wave is not evoked until after cessation of stimulation, and it also demonstrates that while a short train of volleys in the FRA may evoke a substantial late DRP, increasing the length of the train may decrease the amplitude of late wave.

4. Hyperpolarization of primary afferents after DOPA

There is sometimes evidence that after injection of DOPA volleys in the FRA produce a positive DRP. In Fig 8 DOPA as usual depressed the early DRP evoked from the FRA and produced a late DRP. However, interposed between these two components of the DRP there was also a positive potential which was clearly evoked from the FRA since there was hardly any positivity from group I afferents (D) but an increasing effect (in L-G) when the stimulus strength was increased. Record C shows that the positive DRP can also be evoked by a single volley in high threshold afferents, but in this case it was not followed by a late DRP.

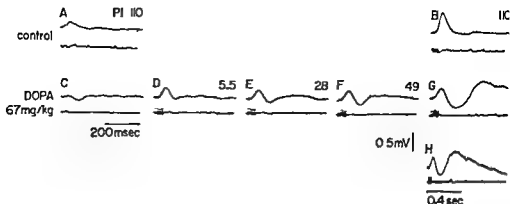


Fig. 8 Positivity in the DRP evoked from the FRA after DOPA. The upper traces show the DRPs; the lower traces are recorded from the dorsal root entry zone. The PI nerve was stimulated with

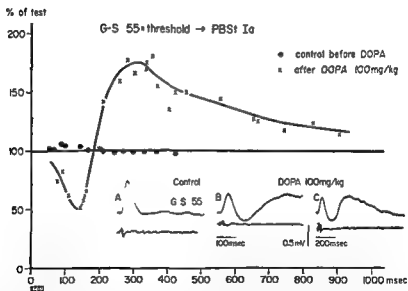


Fig. 9 The correlation after DOPA between the positive and late negative waves in the DRP and the excitability of Ia afferent terminals. Upper traces of records A-C show the DRP evoked by stimulation of the G-S nerve to activate high threshold afferents before (A) and after (B-C) injection of DOPA (100 mg/kg). The lower traces are recorded from the dorsal root entry zone. The graph shows the correlation between the positive and late negative waves in the DRP (cf. curve and C). Time scale the same for A and B. Voltage calibration applies to all DRPs.

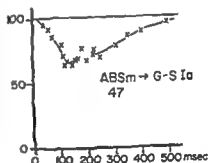
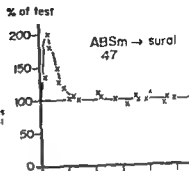
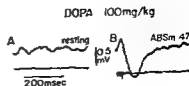


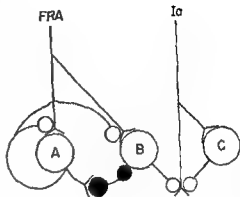
Fig 10 Measurements of the excitability of cutaneous and Ia afferents to an antidromic volley evoked by local stimulation within the dorsal horn.

lower traces are recorded from the dorsal root entry zone. The upper curve shows the time course of the increase in excitability of the terminals of cutaneous afferents of the sural nerve when conditioned by volleys in the high threshold afferents of the ABSm nerve plotted as in Fig 2. The test antidromic volley was evoked by local stimulation within the dorsal horn. The lower curve shows the excitability changes of Ia afferent terminals of the G-S nerve with the same conditioning volleys. The test antidromic volley was evoked by local stimulation within the G-S motor nucleus. The decreased excitability corresponds well in time to the positivity in the DRP (B). Same time scale for A and B, voltage calibration applies to the DRPs.

Excitability measurements have shown that the positive DRP is associated with a decreased excitability in Ia afferent terminals. In Fig 9 a train of volleys in high threshold muscle afferents gives an initial decrease in the Ia excitability followed by the later long lasting increase. On the other hand, no decrease in excitability was observed in Ib or cutaneous afferents during the positive DRP.

Fig 10 is from an experiment in which the positive DRP evoked from high threshold muscle afferents was exceptionally large and not followed by a late negative wave. The excitability measurements in the lower curve show a long lasting decreased excitability in Ia afferents whereas in cutaneous afferents upper curve there is only the early increased excitability, with no evidence of a later decrease in excitability. Since there was no late negative wave in this experiment it is assumed that record B and the lower curve give the time course of the primary afferent hyperpolarization when not interrupted by the late PAD. Fig 11 is from another experiment in which again there was a predominantly positive DRP evoked from the FR after DOPA had been injected (A and B). The group I volley from PBSt had separation into Ia and Ib components (C), and the antidromic volley evoked

Fig. 12 A schematic representation of the neuronal pathways involved in the depolarization of Ia afferent terminals evoked by stimulation of the FRA. Pathway A is activated in the acute spinal cat and inhibits pathway II so that normally no depolarization of Ia terminals results from activation of the FRA. The black interneurone is inhibitory but it is not known if it operates postsynaptically or presynaptically by depolarizing the terminals of interneurons. Pathway A can be partially or completely suppressed by injection of DOPA.



for the depolarization of Ia terminals evoked by activation of Ia afferents, it may share interneurons with pathway II (see Discussion). A single interneurone in the diagram may represent a chain of interneurons.

terminals (Eccles 1964). This selective action is completely reversed during the late long lasting DRP evoked from the FRA after iv injection of DOPA only. Ia afferent terminals (from flexors and extensors) are depolarized and there is no effect on Ib or on cutaneous afferent terminals. To judge from the size of the DRP and the excitability increase this PAD from the FRA may be as large as that evoked by volleys in group I afferents from flexors (Eccles, Magni and Willis 1962). The latter action gives presynaptic inhibition of transmission from Ia afferents (Frank and Fuortes 1957, Eccles, Eccles and Magni 1961) and it is assumed that the PAD evoked from the FRA also has an inhibitory effect on transmission from Ia afferents. The functional significance of this inhibitory action in motor regulation will be considered in a forthcoming paper on the late reflex activation of motoneurons, which can be evoked after injections of DOPA (cf Jankowska *et al* 1965, Lundberg 1965, 1966). The main object of the following discussion is to consider why volleys in the FRA have no effect on Ia afferents in the normal acute spinal preparation and how it comes about that there is an effect after DOPA has been given. It is postulated that this problem is essentially one of inhibitory interaction between spinal reflex pathways from the FRA.

The neuronal linkage between the FRA and primary afferent terminals is shown schematically in Fig. 12. Without DOPA volleys in the FRA give a PAD in their own terminals (Eccles, Kostyuk and Schmidt 1962) through the interneuronal pathway marked A. B indicates the interneuronal pathway from the FRA to Ia afferents disclosed by DOPA and which is responsible for the long latency PAD. This scheme makes no attempt to account for the fact that excitatory and inhibitory effects of very different latencies and durations may be produced; this problem will be discussed in a later paper. Our results indicate that there is no transmission in pathway II when A is active and *vice versa* no transmission in pathway A when II is active. This interrelationship suggests an inhibitory interaction between pathway A and B. One possibility would be that DOPA facilitates pathway B (which will

not function without a facilitatory support) and that when this pathway is active it inhibits pathway A. However, the alternative linkage of an inhibition from A to B is shown in Fig. 12, this hypothesis is preferable for the following reasons: 1) DOPA may occasionally depress the short latency path A without opening pathway B, hence the depression of A does not seem to depend on activity in B. 2) On increasing the stimulus strength it is sometimes observed that the effect via pathway B is decreased (Fig. 5 and 6), 3) During prolonged repetitive stimulation of the I RA transmission in B is depressed (Fig. 7). All these observations would be expected with the direction of inhibitory interaction indicated in the diagram of Fig. 10 but are difficult to explain by an inhibition from B to A.

It has been suggested that DOPA modifies the effects of volleys in the I RA by liberating transmitter from a descending noradrenergic pathway, which inhibits transmission in the short latency paths from the I RA (Andén *et al.* 1966 a, b). Engberg and Ryall (1965, 1966) have postulated that noradrenaline is an inhibitory transmitter in the spinal cord and that the descending noradrenergic pathway inhibits transmission in the short latency I RA pathway by a monosynaptic action. It is now suggested that DOPA acting through the descending noradrenergic system inhibits transmission in pathway A and in doing this removes the inhibitory effect on pathway B thereby permitting transmission via this pathway from the I RA to Ia afferent terminals. It is necessary to emphasize the tentative nature of the hypothesis that DOPA acts via a descending noradrenergic pathway (cf. Andén *et al.* 1966 b), further investigations are required to establish the exact mechanism of its action, but a detailed knowledge of the mechanism by which DOPA acts is not required for the discussion of the main problem which is the interaction between paths A and B in Fig. 12. The important factor in releasing path B seems not to be that DOPA is given but that transmission in the short latency path A is depressed. This is indicated by our finding that a late PAD in Ia afferents was evoked from the I RA in two spinal cats to which DOPA had not been given but in which transmission in the short latency pathways from the I RA was depressed for unknown reasons. It should however not be assumed that any depression of the short latency path from the I RA will necessarily give a release of the pathway from the I RA to Ia afferents. For example there is usually no release of transmission in the latter pathway during the tonic decerebrate inhibition of transmission in the short latency paths from the I RA (Eccles and Lundberg 1959, Carpenter *et al.* 1963). The explanation may be either that in this state there is descending inhibition both of pathway A and B or else that the first interneuronal relay (not shown in Fig. 12) is common for both these pathways and that the decerebrate inhibition is exerted at this level. If so, the release of transmission from the I RA to Ia afferents should only occur when the second order interneurons in the short latency path from the I RA are inhibited. The reason why it is important to consider different schemes for the interneuronal linkages from the I RA is the accumulating evidence that there are several descending pathways controlling transmission from the I RA (Lundberg 1966). Because of the interaction between the various interneuronal reflex pathways the significance of a

descending facilitatory or inhibitory system may depend entirely on where in the interneuronal chain the effect is exerted.

According to Fig. 12 interneurons in path II receive both excitation and inhibition from the FRA. In recent experiments intracellular recording from interneurons has demonstrated that many interneurons receive *postsynaptic* inhibition from the FRA, convergence of excitation and inhibition from the FRA was observed in several interneurons (Hongo, Jankowska and Lundberg 1965, 1966) but there may probably also be *presynaptic* inhibition through depolarization of synaptic terminals from interneurons (cf. Lund *et al.* 1965). Inhibition of pathway B is also of interest in relation to the long latency of transmission from the FRA to Ia afferents. Transmission in pathway A is presumably only partially inhibited by DOPA and a synchronized volley in the FRA may delay transmission through pathway B by means of the inhibition exerted from A in Fig. 12. The observation that an increased strength of stimulation may prolong the latency of the PAD in Ia afferents supports this interpretation. This interpretation implies that the inhibition of the late DRP (Fig. 5 and 6) is an indication of the inhibitory mechanism that normally prevents the production of the late PAD in Ia afferents.

The inhibitory effects are probably also significant in relation to the selective hyperpolarizing action in Ia afferent terminals that volleys in the FRA may have after DOPA (Fig 8—11). If for some reason there is a continuous activity in pathway B the Ia afferent terminals may be kept in a depolarized state. Under such conditions the inhibitory effect from the FRA via pathway A may stop the activity in pathway B and thereby repolarize the Ia afferent terminals. Similar mechanisms have been proposed as explanations for the primary afferent hyperpolarizations evoked from C fibres (Mendell and Wall 1964) and from the brain stem (Lundberg and Olsson 1963, 1966).

One problem remains to be discussed in relation to inhibition of transmission to Ia afferents. Fig. 12 C shows the pathway by which Ia afferents may produce PAD in Ia terminals. Transmission in this pathway can be very effectively inhibited from the FR A (Lund *et al.* 1965) this inhibitory effect is not indicated in Fig. 12, where B and C are shown as separate pathways, but they may have interneurons in common and, if so, it should be considered if the inhibitory effect of Lund *et al.* (1965) on pathway C may be identical with that exerted on pathway II in Fig. 10. In this connection it is of interest that also the inhibitory effect of Lund *et al.* (1965) is depressed after DOPA.

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The Effect of DOPA on the Spinal Cord

4 Depolarization Evoked in the Central Terminals of Contralateral Ia Afferent Terminals by Volleys in the Flexor Reflex Afferents

By

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Abstract

JANAKOWSKA, E., S. LUND and A. LUNDBERG The effect of DOPA on the spinal cord 4 Depolarization evoked in the central terminals of contralateral Ia afferent terminals by volleys in the flexor reflex afferents Acta physiol. scand. 1966 68 337-341

After injection of DOPA in unanaesthetized spinal cats volleys in the flexor reflex afferents (FRA) evoke a late ipsilateral dorsal root potential (DRP) which is caused by a selective depolarization in the central terminals of Ia afferents (Anden *et al.* 1966a, b). In the present paper it will be shown that after DOPA volleys in the FRA produce similar effects on the contralateral side. The experimental procedures have been given in the previous papers.

Results

The DRPs in Fig. 1 are evoked by short trains of stimuli. After an intravenous injection of DOPA the short latency DRPs evoked from contralateral cutaneous afferents (I) and high threshold muscle afferents (I-J) are depressed (cf. Anden *et al.* 1966a), but volleys in these afferents now produce a late long lasting DRP, which resembles that evoked on the ipsilateral side (Anden *et al.* 1966a, b). The contralateral late DRP was never evoked by volleys in group I muscle afferents but could sometimes be produced from low threshold group II afferents (record 11).

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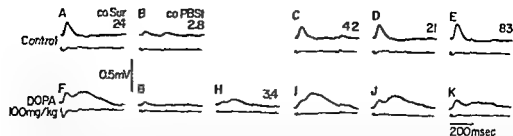


Fig 1 The effect of DOPA on the DRP evoked from the contralateral FRA. The upper traces are DRPs recorded from the most caudal dorsal rootlet in L6; the lower traces are recorded from the L7 dorsal root entry zone. Records A—E were obtained before and F—K after an intravenous injection of DOPA (100 mg/kg). The contralateral sural nerve (co Sur) was stimulated in A and

same time scale for all records

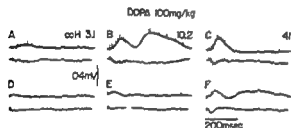


Fig 2 As in Fig 1 but recording after DOPA had been given and stimulation of the contralateral hamstring nerve (co H).

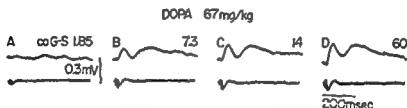
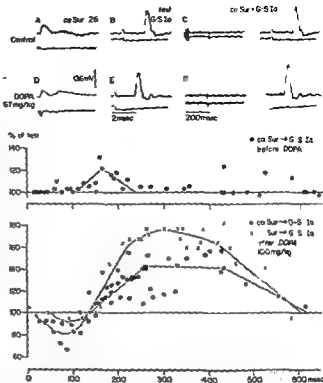


Fig 3 As in Fig 1 but recording after DOPA had been given and stimulation of the contralateral gastrocnemius-soleus nerve (co G-S).

although in many experiments it was necessary to stimulate group III afferents. In record I (Fig 1) a maximal contralateral late DRP was evoked at a strength of 4.2 times threshold and when the stimulus strength was raised there is an increase of the latency and a decrease of the magnitude (J and K). An even more pronounced reduction with increased stimulus strength is shown in Fig 2 in which a late DRP is evoked in B and abolished in C when the stimulus strength is raised from 10.2 to 41 times threshold. The corresponding lower records (E and F) show that with single stimuli of the same strengths the late DRP appears when the strength is raised from 10.2 to 41 times threshold. The opposite effects in B, C and E, F support the assumption that volleys in the same afferents can produce and inhibit the late DRP (cf Anden *et al* 1966 b). Fig 3 illustrates an experiment in which the late

Fig 4 Measurement of the change in excitability of Ia afferent terminals by volleys in contralateral cutaneous afferents before and after DOPA. The upper traces in A and D are DRPs recorded as in Fig 1, the lower traces in all records are from the L7 dorsal root entry zone. The upper traces in B, C, E and F show the antidromic volley recorded in the G-S nerve when its Ia afferent terminals were stimulated through a microelectrode, whose tip lay in the G-S motor nucleus. For each curve the abscissa shows the interval between the first conditioning volley and the test volley, the ordinate the percentage change in the amplitude of the conditioned antidromic volley, taking the unconditioned response as 100%. A-C and the upper curve were obtained before DOPA, D-F and the lower curves after an intravenous injection of DOPA (100 mg/kg). B and E show the unconditioned test response in Ia afferents. The effect of a train of conditioning volleys in the co Sur is shown by the right hand records in C and F. Conditioning testing interval is shown by the simultaneously obtained slow left hand records in C and F. Observe that before DOPA there is a small late wave in the DRP and a corresponding slight increase in the Ia excitability and that these effects increase markedly after DOPA. For comparison the lower curve shows the effect (x) of the same train of volleys in the ipsilateral sural nerve (i Sur). The voltage scale refers to the DRPs. The cord dorsum record in III was taken at a higher amplification than in A. The time scale for A, D and left hand records in C and F is given in F and for the other records in E.



DRP did not decrease when the stimulus strength was raised to activate high threshold group III muscle afferents.

Wall's (1958) technique of measuring the excitability in afferent terminals was employed to find out in which afferents the late depolarization is evoked on the contralateral side. It was a consistent finding that the excitability increases in Ia afferents (flexors and extensors). Fig 4 shows measurements, before and after DOPA, on Ia terminals in the G-S motor nucleus. In this case volleys in the contralateral sural nerve evoke a slight late increase of the excitability even before DOPA and correspondingly there is also a small late wave in the DRP (A). After DOPA the late wave is more pronounced and the conditioning volleys in the contralateral sural nerve now give a larger increase in the excitability which follows the initial decrease (●). For comparison is also shown the effect evoked from the ipsilateral sural nerve (x). A contralateral small late DRP and a corresponding increase in the Ia excitability was found rather often in acute spinal cats which had not been injected with DOPA. Similar findings were occasionally

DOPA 100mg/kg

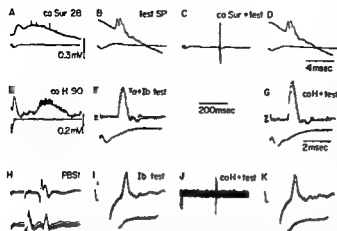


Fig 5 Measurements of the excitability of cutaneous Ia and Ib terminals when conditioned by volleys in the contralateral FRA after DOPA. A and E are DRPs recorded as in Fig 1. The lower traces in all records and also the upper trace in H are from the L7 dorsal root entry zone. The antidromic test discharge (B-D) is recorded in the cutaneous superficial peroneal nerve (SP) and evoked from a microelectrode, whose tip lay in the dorsal horn where a maximal extracellular focal potential was recorded from the SP nerve. The unconditioned test is shown in B; a train of conditioning volleys in the co Sur has no effect (D). The conditioning testing interval is

shown in C. In E-K obtained in another experiment the tip of the intraspinal stimulating electrode was in the intermediate nucleus and the antidromic volley recorded in the PBSt nerve. G shows the facilitatory effect of conditioning volleys in the co H at the same strength as in E (unconditioned group I test in F). In I-K, a maximal Ia volley from the PBSt nerve is collided with the anti-

the ipsilateral side (Anden *et al.* 1966b) but it is not known why, without DOPA the late effect to Ia afferents is more frequently encountered in contralateral than in ipsilateral Ia afferents. Crossed effects to Ia afferents from the FRA were not found by R. M. Eccles, Holmqvist and Voorhoeve (1964a) but this is not surprising since they worked on preparations under Nembutal anaesthesia and the late waves are completely abolished by 5 mg Nembutal i.v.

Fig 5 illustrates excitability measurements from Ib afferents and from cutaneous afferents. B shows the unconditioned test discharge evoked by intraspinal stimulation of cutaneous afferents. Conditioning volleys in a contralateral cutaneous nerve evoke a late DRP (A) but does not change the test discharge (B). F shows the unconditioned antidromic group I volley evoked from the intermediate nucleus. When conditioned by volleys in high threshold muscle afferents the test discharge is increased in G. This increase is due to the raised excitability in Ia afferents because when the collision technique is employed to evoke Ib test the same conditioning volley has no effect (I and K).

Discussion

In the normal acute spinal cat volleys in the FRA evoke an early contralateral DRP which is caused by a PAD in cutaneous afferents (probably in the FRA) and to some extent in Ib afferents, while there is no depolarization in Ia afferents

(R M Eccles *et al* 1964a, b) After injection of DOPA the early contralateral DRP from the FRA is depressed (Andén *et al* 1966 a) and volleys in the FRA evoke a late long lasting contralateral DRP (Fig 1—3), which resembles that recorded on the ipsilateral side (Andén *et al* 1966 b) This contralateral late DRP is caused by a depolarization in Ia afferent terminals and there was no trace of a depolarization in Ib and cutaneous afferent terminals Apparently there is a selective pathway from the FRA not only to ipsilateral (Andén *et al* 1966 b) but also to contralateral Ia afferents Since also the short latency effects from the FRA are bilateral (R M Eccles *et al* 1964 a, b) the generalizing postulate is forwarded that the effects from the FRA to primary afferents are bilateral

Evidence has been presented (Fig 1 and 2) showing that the crossed pathway from the FRA to Ia afferents is under the same inhibitory influence from the FRA as the corresponding ipsilateral pathway (Andén *et al* 1966 b) By analogy it is suggested that without DOPA also the crossed pathway is inhibited by activity in the short latency path from the FRA and that DOPA by depressing transmission in the latter path releases the long latency path from this inhibitory control

It will be shown in a forthcoming paper in this series that knowledge of the nature of the contralateral late DRP is essential for the understanding of the mechanism for reciprocal activation of flexor and extensor motoneurons from the FRA after DOPA (cf preliminary report by Jankowska *et al* 1965 Lundberg 1965 1966)

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Monoamine Oxidase Activity in the Bovine Splenic Nerve Granule Preparation

By

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Abstract

ROTH, R. H. and L. STJÄRNE *Monoamine oxidase activity in the bovine splenic nerve granule preparation* Acta physiol. scand. 1966. 68. 342—346

Monoamine oxidase (MAO) activity was determined in fractions obtained from bovine splenic nerve with both dopamine (DA) and noradrenaline (NA) as substrates. DA was found to be a much better substrate for MAO than NA. The bulk of the MAO in this preparation was found in the nerve granule fraction which by electron microscopic evaluation seemed to be largely free from mitochondrial contamination. These findings suggest that bovine splenic nerve in addition to mitochondrial bound MAO also contains a form of MAO with a substrate preference for DA which seems to be associated with the nerve granules.

In work currently under progress in this laboratory, DA- 3 H has been routinely used to quantitatively and qualitatively evaluate the β -hydroxylation step in the synthesis of NA in bovine splenic nerve granules (Stjärne, Lishajko and Roth, 1966). It was therefore considered desirable to also investigate the relative activity of MAO present in this preparation, since the labeled amine might be degraded to some extent by this enzyme. It has been previously assumed that little MAO activity is present in the bovine splenic nerve granule preparation since balance studies involving the release of NA from nerve granules indicated that most of the NA lost from the granules over a 1 hour period at 20 °C was recovered as intact NA in the supernatant (Euler, 1961). However, it is also known that DA is a much better substrate for MAO than is NA (Weiner, 1965) and therefore MAO may play a more prominent role in regulation of the level of this monoamine in bovine splenic nerve.

The present investigation indicates that not only is there substantial MAO activity present in bovine splenic nerve but that a large portion of this activity appears to sediment with the granule fraction.

Methods

Fresh bovine splenic nerve tissue was obtained from the slaughter house, carefully dissected free from contaminating tissue, and homogenized at 0—5 °C in isotonic po-

potassium phosphate pH 7.5 (10 g/30 ml), either by means of an Ultra Turrax apparatus or by squeezing between nylon rollers. The whole nerve homogenate is pressed juice was then centrifuged at $9\,000 \times g$ for 10 min and the pellet discarded. The low speed supernatant which contains the specific NA storing nerve granule was used for the experiments. In some cases the granules were separated by centrifuging the supernatant at $50\,000 \times g$ for 30 min. The granules obtained were then resuspended in an equal volume of isotonic potassium phosphate.

One to 2 ml of the above prepared granules or supernatant — granules were incubated at 37°C in a centrifuge tube for varying time intervals and with different substrate concentrations of NA and DA. In all experiments the tubes contained $1\ \mu\text{C}$ of tritium labeled DA (New England Nuclear Corp., 3,4-dihydroxyphenylethyl- 1-H^3 amine HBr, specific activity = $50\ \text{mc/mM}$) or $1.5\ \mu\text{C}$ of H^3 di NA (New England Nuclear Corp., purified prior to use by ion exchange column chromatography, specific activity = $7\ \text{c/mM}$). The reaction was terminated by the addition of $0.2\ \text{ml}$ of $2\ \text{M}$ perchloric acid and the tubes were placed in an ice bath. The suspension was neutralized to about pH 6 by addition of potassium carbonate and then centrifuged at $30\,000 \times g$ for 10 min at 0°C to remove the precipitated protein and perchlorate. The supernatant was then decanted into stoppered glass tubes containing $200\ \text{mg}$ of Dowex 50. The tubes were shaken in a mechanical shaker for 15 min to assure quantitative adsorption of all the amines and then centrifuged at $2\,500\ \text{RPM}$ for 5 min to obtain a clear supernatant. One hundred μl of the supernatant which contains the deaminated metabolites was taken for radioactive determination. Control tubes were precipitated with perchloric acid before addition of the radioactive substrates and then carried through the identical procedure. The radioactivity was determined by counting the samples in a Packard Tri Carb liquid scintillation spectrometer. The scintillation mixture consisted of a 7:3 toluene absolute ethanol solution containing $4\ \text{g}$ of 2,5-diphenylloxazole and $100\ \text{mg}$ of 1,4-bis[2-(4-methyl-5-phenylloxazolyl)]benzene/liter of toluene. Quenching was monitored by internal standards.

Results

Preliminary experiments employing C^{14} tryptamine for MAO determination according to the method of Wurtman and Axelrod (1963) in homogenates from bovine splenic nerve prepared by the Ultra Turrax method demonstrated that about 60% of the MAO in this preparation was present in the low speed supernatant. Further experiments were undertaken to clarify this finding.

Initially experiments were run to determine how long the MAO activity remained linear. When either DA or NA was used as a substrate ($160\ \text{nmoles/ml}$) it was found that the MAO activity in the low speed supernatant preparation remained linear for at least 16 min. A shorter time interval of 8 min was chosen to study the effects of increasing substrate concentration on the rate of deamination. Fig. 1 shows the results obtained with splenic nerve low speed supernatant. In this preparation the MAO seemed to be saturated at a substrate concentration of $320\ \text{nmoles/ml}$ when either DA or NA was used as a substrate. The rate of deamination of NA

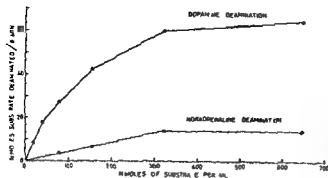


Fig 1 Nmoles of dopamine or noradrenaline deaminated by splenic nerve low speed supernatant incubated for 8 min at 37° C in the presence of various concentrations of substrate

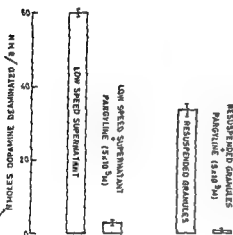


Fig 2 Monoamine oxidase activity (expressed as nmoles of dopamine deaminated/8 min) in fractions obtained from Ultra Turrax treated bovine splenic nerve both in the absence and presence of Pargiline 5×10^{-4} M. Vertical bars depict the range of three determinations

strate concentration of 320 nmoles/ml (i.e. when the enzyme appears to be saturated) was only about 1/5 of the rate of DA deamination.

The resuspended granule fraction contained over 50% of the MAO activity of the low speed supernatant (cf. Fig. 2). Pargiline (Eutonyl®) at a concentration of 5×10^{-4} M was found to be a potent inhibitor of the MAO activity in both systems studied. At this concentration the inhibitory effect was

These results are summarized in Fig. 3. The granule fraction obtained by this procedure was also taken for electron microscopic analysis. According to electron microscopic evidence (cf. Fig. 4) this preparation appears to be strikingly homogenous and apparently largely free from contamination with mitochondria. The results on the distribution of MAO obtained with this pressed juice preparation are similar to those obtained with the fractions prepared by the Ultra Turrax method and again demonstrate that a large percentage of the MAO activity is bound in a particulate fraction which appears morphologically different from mitochondria.

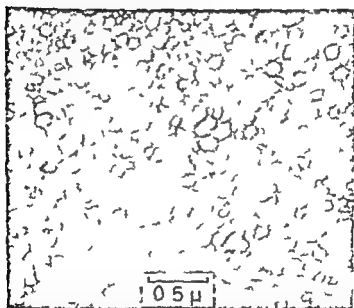
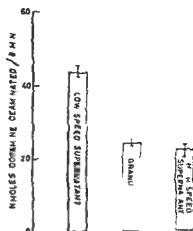


Fig. 4. Electron micrograph (Euler, Shanley, and Lishajko, personal communication) of the high speed sediment obtained by centrifugation of the press juice of Euler 19.8 at 50,000 g for 30 min after previous removal of the coarse fraction by centrifugation at 9,000 g for 10 min.

Discussion

The present experiments demonstrate that the granule fraction obtained from bovine splenic nerve contains a substantial amount of MAO activity. The MAO present shows a substrate preference for DA over NA despite the fact that DA/H₂O₂ was used to measure the activity. It would be expected that some isotope effect should be apparent with this substrate (Belleau and Morin, 1963) and therefore the rate

deamination estimated for DA is probably at best a low estimate of the actual rate of deamination of unlabeled DA.

Blaschko, Hagen and Hagen (1957) have demonstrated that the majority of the MAO is mitochondrial bound and can be separated from the amine granules by density gradient centrifugation. The electron microscopic observations on the bovine splenic nerve granule fraction, however, seems to suggest that the MAO that follows the granule fraction is not present in normal mitochondria, since these are almost completely absent in this preparation. However, the possibility still remains that some sort of specialized mitochondria exist in this preparation with morphology similar to that of the amine containing granules. Further investigations are now under progress in this laboratory in an attempt to relate the NA, ATP, protein, succinic dehydrogenase, β -hydroxylase and MAO content of the various fractions of bovine splenic nerve homogenates obtained by density gradient centrifugation.

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Length at Inactivated Contractile Elements, Length-tension Diagram, Active State and Tone of Vascular Smooth Muscle

By

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Abstract

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Analysis of the mechanical properties of vascular muscle is complicated by the fact that it is small, it is contracted to a certain degree and has no constant resting length. By total blockade of the energy production of the muscle with moniodoacetate acid under anaerobic conditions the vessel length at inactivated contractile elements could be measured. With the aid of this length the length-tension diagram of the vascular muscle in untreated and adrenaline-treated vessels was studied. The muscle developed a maximal tension (2 100 gwt/cm² cross section) at this length which therefore probably corresponded to L_0 in skeletal muscle. The vascular muscle shortened maximally to 7 per cent of the relaxed length. Both the development of maximal isometric tension and the maximal isotonic shortening were of the same magnitude in vascular as in skeletal muscle. The series elastic component (13—20 per cent) was on the other hand greater in vascular than in skeletal muscle. No rigor mortis was noted in the vascular muscle. The spontaneous tension of the vascular muscle and tension increase induced by adrenaline could be largely ascribed to an active state. The question of whether a "move" or "catch" mechanism is present in vascular muscle independent of the active state is discussed.

In studies of the elastic and mechanical conditions of the vessel wall, certain properties are encountered that may be ascribed to its contractile elements and others that may be ascribed to the elastic and collagenous elements. As in the case of skeletal muscle (Hill 1919) it may be appropriate to envisage the mechanical properties of the vessel wall schematically by assuming that it consists of a system with three components: 1) the contractile element, 2) the elastic component combined serially with the contractile element, 3) the elastic component running parallel with the contractile element. The vascular wall has in addition marked viscous properties. None of these elements or components appear to be consistently identifiable with any morphological structures in the vessel wall. The contractile element obviously consists of muscle cells. It is probable that smooth muscle together with

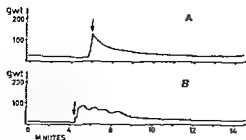


Fig 2A Tension curve for vascular specimen in which the length was quickly increased from 11.0 mm to 13.0 mm. The arrow indicates the time of lengthening.

tion. At the arrow the specimen was lengthened from 11.0 to 12.0 mm, whereby the tension rose to approximately 40 gwt. This was followed by spontaneous rhythmic tension-increasing "stretch response".

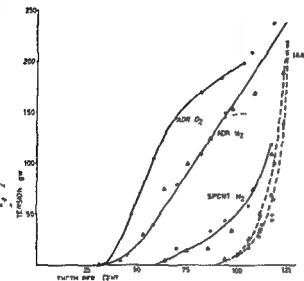


Fig 3 Length-tension curves in three different specimens from the same vessel on successive extension. Adrenaline (A) in a concentration of 1×10^{-6} was added and the maximal tension increase was noted. The adrenaline was then washed out and the specimen was stretched 2 mm. After 30 min A was again added and the procedure was repeated. One specimen was used for each curve.

(O—O and Δ—Δ) were suspended in N_2 -gassed solution (ADR- N_2), the relaxed lengths were 16.0 and 14.5 mm respectively.

The dotted line (ADR N_2) is the tension difference between A and IAA treated muscle (= active tension). Spont N_2 denotes the initial tension in anaerobic specimens before addition of A.

after A is calculated here as approximately 2,100 gwt/cm². The load at relaxed length (10 gwt) was calculated to be 130 gwt/cm².

length of the specimen between the points of attachment was $5 \pm 2 = 7$ mm. The length and tension of the specimen could be determined simultaneously in this way.

The tension in the vascular specimen was measured with a Grass force transducer FTO3 and

Determination of the length-tension curve

The length-tension relationship was determined in untreated and adrenaline (N_1)-treated vascular muscle. The vessel was stretched to a desired length, contracted and the tension was measured. The vessel was then stretched to a new length, contracted and the tension was measured. This process was repeated until the length-tension curve was determined.

development after treatment with A was determined as in point (2), the specimen was extended repeatedly, by 1–2 mm at a time, before A was added. It was only stretched sufficiently for the maximal momentary tension increase to reach 200 gwt. When the tension had decreased and after 10–20 min, had become stable at a lower level, the vessel was again stretched until the desired length was obtained and the tension had stabilized at a new level. In this way attempt was made to eliminate from the calculations the tension induced by viscous resistance of the vascular muscle on changes in length.

The contraction of the vessel was induced by A in a concentration of 10^{-5} this concentration having a maximal tension increasing effect. In order that A should not be oxidized and exert a progressively diminishing effect, several experiments were performed under anaerobic conditions. The maximal contraction increasing effect of A was somewhat greater in oxygen than in N_2 (Fig. 3).

Muscle length at inactivated contractile elements

however, the specimen was allowed to relax overnight and its length was measured about 16 hrs after the addition of moniodoacetic acid. The length-tension curves of the specimens after 4 and 16 hrs' treatment with moniodoacetic acid showed similar courses.

The concentrations of adenosine triphosphate (ATP) and creatine phosphate (CrP) in vascular muscle were determined according to the method of Adam (1962).

Results

The concentration of energy-rich phosphate compounds in untreated and moniodoacetic acid-treated vascular muscle. Vascular muscle which had been immersed in the tissue bath for 30–60 min at 37° C in Tyrode's solution containing glucose and gassed with 93.5 % O_2 + 6.5 % CO_2 , contained on the average ($n = 13$) 0.91 ± 0.10 μ moles ATP/g wet weight and 0.66 ± 0.09 μ moles CrP/g. After treatment of the specimens with 0.022 M moniodoacetic acid for 8–24 hours in Tyrode's solution gassed with N_2 , they were found to contain neither ATP nor CrP. Since with the analysis method used ATP or CrP could be demonstrated in as low concentrations as 0.02 μ moles/g, the concentration of energy-rich phosphate compounds in the moniodoacetic acid-treated specimens was reduced to below 1 % at least, of that in the untreated specimens.

Rigor mortis in vascular muscle. A striated muscle that completely loses its ATP content develops rigor mortis so that tension occurs on stretching of the muscle (Bendall 1960). We have attempted to demonstrate rigor mortis in vascular muscle, but have been unsuccessful. In these experiments maximal isotonic contraction was induced by A in one vascular specimen so that its length was about 30 per cent of the relaxed length. Moniodoacetic acid was then applied to the maximally contracted specimen which was left with no load applied. After 16 hours the length-tension curve was determined. Tension developed at the same length as in the vascular specimen which was already extended to its relaxed length before treatment.

monoiodoacetic acid. No signs of rigor mortis were seen, and in this respect vascular and skeletal muscle thus seem, in principle, to behave differently.

The length-tension curve in untreated and monoiodoacetic acid-treated muscle

Fig. 3 illustrates a typical experiment in which the untreated vascular specimen was slowly extended by 2.0 mm at a time. The tension rose considerably at first, and then rapidly decreased (Fig. 2A). When the viscous resistance of this vascular tissue had been overcome, after 20–30 min, the tension became stable at a higher level, the value of which is given in Fig. 3 as spont-N_2 . The length of the vascular tissue is given in per cent of the relaxed length (see below). With increasing length the tension rose hyperbolically.

The specimen was then shortened so that the tension decreased to zero, after which it was treated with monoiodoacetic acid. The length-tension diagram was then determined 24 hrs later (curve IAA). The length-tension diagram after monoiodoacetic acid was deviated markedly to the right, i.e. towards greater lengths. Another difference was that as long as the monoiodoacetic acid-treated specimen was only loaded with up to 50 gwt, there was no lengthening of the vessel. The tension was constant from the start, and no viscous resistance occurred. However, when monoiodoacetic acid-treated vascular tissue was subjected to tension exceeding 50 gwt, a lengthening occurred and a viscous resistance was noted. On successive extension of the vessel the tension curves even lay above that obtained with subsequent successive shortening, i.e. hysteresis occurred. At 10–20 gwt, however, the curves again converged on repeated extension and shortening of the vessel (Fig. 7).

Muscle length at inactivated contractile elements. At a tension of 10 gwt (about 100–150 gwt/cm² cross section) the monoiodoacetic acid-treated vascular tissue therefore exhibited a constant length which remained unchanged both on successive extension and shortening. The length of the monoiodoacetic acid-treated specimen at 10 gwt has therefore been taken as reference length in the following; it is denoted as 'relaxed length', and other lengths are given in per cent of this measurement.

Length-tension diagram after adrenaline. Fig. 3 shows also a typical experiment where vascular specimens (at different lengths) were contracted with A and the tension was related to the muscle length in per cent of the relaxed length. The effect of A was first determined on the maximally contracted specimen, after which the specimen was extended successively before each new addition of A. Three different specimens from the same vessel were studied in this experiment, two under anaerobic and one under aerobic conditions. The spontaneous tension, i.e. the tension before the addition of A, of the two anaerobic specimens is also given in Fig. 3. The difference between the curves for spontaneous tension and tension after addition of A gives the increase in tension which can be ascribed to A. Also shown in Fig. 3 is the length-tension diagram after treatment of the specimen with monoiodoacetic acid. It is highly probable that the tension differences between the curves for 'monoiodoacetic acid', on the one hand, and "spontaneous tension" and "A", on the other, can be ascribed to the contractile elements. As seen in the figure, the tension increase after A was

Fig 4 Length tension diagram of two specimens from the same vessel. In one of them (○—○) the spontaneous tension and the tension after addition of Δ were recorded on successive extension of the specimen. In the other the tension on successive shortening was recorded (○—○)

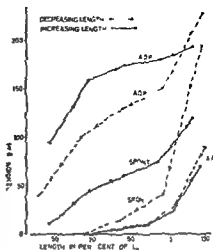
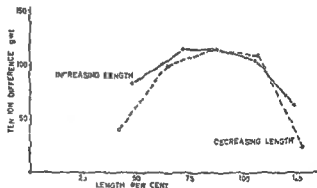


Fig 5 Difference in tension between Δ induced and spontaneous tension in the experiment illustrated in Fig 4



maximal at the relaxed length, when it reached a value of approximately 2,000 gwt/cm² cross-section. In skeletal muscle the maximal tension development is approximately 2,000–3,500 gwt/cm². Wilkie 1954 Ramsey 1960. At 25–30 per cent of the relaxed length vascular muscle is no longer exert any tension but seems to be maximally contracted. In other respects the length tension curve after treatment with Δ followed the same course as has been described for skeletal muscle (Wilkie 1954) and smooth muscle such as rabbit uterus (Sapo 1960) and taenia coli (Valberg and Axelsson 1963). The maximal shortening of the vascular muscle in per cent of the relaxed length was the same as has been reported for skeletal muscle in per cent of L_0 Ramsey 1960.

Fig 4 illustrate an experiment in which two different specimens from the same vessel were studied one on successive extension and the other on successive shortening. The length tension curve with stepwise extension lay considerably above that with successive shortening. When the tension increasing effect of Δ was determined in relation to the spontaneous tension, however, the increase was found to be almost the same in the two specimens (Fig 5). The curves showed a level maximum at 75–100 % of the relaxed length.

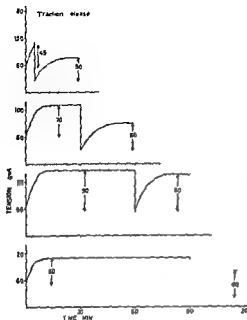


Fig 6 Active state determined by traction release. Adrenaline (1×10^{-6}) was added to all specimens at time 0. After 30, 60 and 90 min the specimen was extended 2 mm but so slowly that the tension increase never exceeded 200 gwt. When the tension had begun to diminish the length of the vessel was rapidly adjusted. The increase of active tension after 30 min was recorded. The figures denote the tension increase in gwt after treatment with A before and after traction release.

Determination of active state. In attempts at determining the active state of vascular muscle we first tested the "quick release" method of Ritchie (1934). With this method the isometrically contracted muscle was shortened 1–2 mm by tightening the micrometer screw 1–2 mm within 1 second so that the tension decreased to zero. These vessels had a maximal isotonic shortening velocity of approximately 1 mm/min. It was not necessary therefore to "release" the muscle very rapidly for avoidance of active shortening. The extent to which the muscle regained its original tension was then observed. This procedure was usable but had certain disadvantages. In vascular muscle the series elastic component was so large that the muscle had to shorten about 2 mm (15–20 per cent of the relaxed length) for the tension to decrease momentarily to 0 gwt. In skeletal muscle the series elastic component is reported by Hill (1940) to be 3–4 per cent of l . At the shorter length the maximal tension development of the vascular muscle was considerably reduced (cf. Fig. 3) in comparison with the greater length. The muscle never therefore regained its original tension after quick release. With this method the existence of an active state could be demonstrated but its intensity could not be determined. In an attempt to eliminate this source of error the "quick release" technique was combined with preliminary extension of the muscle, and the active state was determined by "traction release." With this method the muscle was extended initially 1–2 mm. This was done over the course of 1–2 mins and care was taken that the tension should not exceed 200 gwt (Fig. 6). When the decrease in tension had begun to diminish and the tension was only a little greater than before the muscle was lengthened "quick release" (1–2 mm) to the original muscle length was performed. With this the tension fell to a level below

the original but then rose, first rapidly and then more slowly to a high constant level after 10–20 mins (Fig 6). The increase in tension after traction release was assumed to reflect the degree of active state of the muscle.

The risk with this procedure was that extension of the muscle might provoke a stretch response and create an active state where none existed previously. However it seems to be difficult to provoke a stretch response in these vessels. Under normal conditions the tension never rose above the level that was induced by the extension and the final tension was not reached until 20–30 min after this extension. In experiments where the muscle was suspended for 24 hrs in a free Tyrode's solution however a stretch response was induced. Extension of the muscle after this treatment induced an active tension increase which markedly exceeded that induced by extension (Fig 2 B).

Another question was whether traction release in any way reduced the reactivity of the muscle. One isolated traction release did not affect the vascular muscle. The contractile effect of A was the same before as after a traction release. In this case A was washed out between the experiments. With repeated traction release at the same muscle specimen in the constant presence of A however the development of active tension successively diminished.

Active state in the vascular muscle. It seemed highly probable that the difference in tension between A treated and monoiodoacetic acid treated muscle could be ascribed to the contractile element. But was this tension due to an active state or was it dependent on a tonic or catch mechanism in the vascular muscle? This problem was of especial interest since in previous experiments on vascular muscle we had found that on isometric contraction induced by A the energy requirement was 3–4 times greater during the phase of contraction in which tension was being increased than when a constant tension level had been reached (Lundholm and Möhne Lundholm 1963). On investigating the energy requirement on isotonic contraction of the vascular muscle we found that this requirement increased during the shortening phase and then returned to the basal value (Lundholm and Möhne Lundholm 1962) in spite of persistence of the isotonic contraction. If a parallelism was assumed between the energy requirement and the intensity of the active state these results could indicate that during the later stage the degree of active state had decreased and a catch mechanism was possibly in operation.

The degree of active state with continuous influence by A is shown in Fig 6. After traction release the increase in tension from the initial value after 6, 30, 60 and 90 mins treatment with A was of the same magnitude as the initial tension increase on addition of A. The absolute tension developed by the muscle was however lower after traction release when the initial tension lay at a lower level. The results indicate however that the degree of active state was unchanged on continuous treatment with A.

Another interesting question was whether the spontaneous tension noted in the muscle was a consequence of active state or was due to a more passive mechanism.

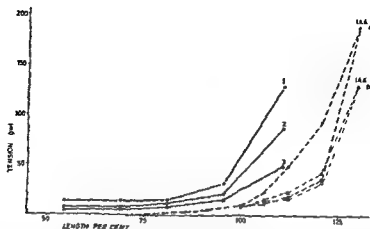


Fig 7 Spontaneous tension and active state. Curve 1: spontaneous tension of the vascular specimen on successive extension by 2 mm each time. Curve 2: the tension finally developed by the specimen 30 min after traction release. The tension differences between (2) and (3) may be ascribed to an active state. After monoiodoacetic acid treatment two consecutive length tension determinations were made (a and b).

Traction release was therefore also performed on untreated vascular muscle at different lengths in an attempt to answer this question. The results are shown in Fig 7. In this figure, "1" is the initial spontaneous tension, "2" the tension regained by the muscle after traction-release and "3" the lowest tension exhibited immediately after release. The differences between "2" and "3" should therefore reflect that part of the spontaneous tension that resulted from an active state. As may be seen, the spontaneous tension could be ascribed partly, but not wholly, to an active state.

Discussion

Relaxed length of inactivated contractile elements. One important question in evaluating the significance of relaxed length was whether monoiodoacetic acid in the concentration used only influenced the metabolism and thereby the contractile state of the vascular muscle, or whether it also affected the elastic properties of the vessel wall by means of a physico-chemical process. Several factors support the former idea. 1) the final length attained by the muscle when loaded isotonically during treatment with monoiodoacetic acid was the same as that observed when the metabolism was inhibited by total lack of substrate. 2) in *taenia coli* a relaxed length can be induced by total inhibition of the electrical activity of the membrane with A. Monoiodoacetic acid did not cause further lengthening of adrenaline treated *taenia coli* (Aaberg and Axelsson 1965). 3) normal veins from man were relaxed by monoiodoacetic acid, varicose veins which almost completely lacked smooth muscle were not relaxed by this substance (Jonsson and Ahrenander 1963).

It seems probable that monoiodoacetic acid relaxed the vascular muscle by inhibition of the metabolism, and that the length tension curve obtained here reflected the conditions in vascular muscle where the contractile elements were inactivated.

It should be possible in an inactive muscle to establish a certain reference or resting length. But, as seen in Fig 3 and 7, this length depended on the tension applied to the muscle. The length at a load of 10 gwt (approximately 130 gwt/cm² cross section) was chosen in view of the fact that at this length and tension no hysteresis occurred on repeated extension and shortening. At this length the isometric tension was at its maximum (dotted line Fig 3) and agreed in this respect with L_0 in skeletal muscle (Ramsey 1960).

Active state If the length-tension curve after treatment with monoiodoacetic acid reflected the conditions of the inactive muscle, the vascular muscle under normal conditions is always contracted to some extent. But did the contracted state of the muscle result from an active state alone? The increase in tension after treatment with A was probably due to an active state since the muscle regained its tension after traction-release. The energy utilization was also increased when the muscle contracted isometrically after A (Lundholm and Mohme-Lundholm 1965).

The absolute level reached by the tension after treatment with A was dependent, however, on the initial spontaneous tension of the muscle (Fig 4). The spontaneous tension and the tension resulting from A treatment appeared to be combined. The spontaneous tension was, in turn, probably due in part to an active state, especially at greater lengths (Fig 7). This indicates that the active state induced by A, and the spontaneous active state of the muscle, would be added together. This supposition that active state of different origin may be added is not improbable. When the concentration of A in the suspension solution of the vascular muscle was successively increased until further increase in concentration gave no rise in tension, a further tension increase could be induced by histamine and/or K⁺ ions.

The interesting question of whether the degree of active state in these experiments was due to different degrees of activation of the individual muscle cells or to the fact that different numbers of muscle cells were maximally activated, will be discussed in a later paper.

Another question was whether the increase in tension of the contractile elements on successive extension of the vascular muscle could be ascribed to an active state alone or whether a more passive tonic or catch mechanism was a contributory cause. We refer here to the tension shown by the muscle in a steady state after 30 min. It is seen in Fig 4 that the tension reached by the muscle at a certain length during treatment with A was considerably higher in the experiments where the muscle was successively extended than when it was successively shortened. On traction release of untreated and A treated muscle (Fig 6) the tension level before traction was higher than that finally attained by the muscle after release. These experiments may indicate the presence of a 'catch' mechanism.

The monoiodoacetic acid treated muscle showed the same tension on extension and shortening as long as the maximal tension did not exceed 50 gwt. The monoiodoacetic treated vessel did thus not show viscous properties in this tension region. When the series elastic elements was eliminated there was no 'catch' mechanism present. With higher tensions, however, there appeared a viscous resistance.

tension difference on extension and shortening of the monoiodoacetic vessel which was reflected in the hysteresis of the length tension curve (Fig. 7)

At a higher tension than 50 gwt it is probable therefore that plastic length changes occur in the series elastic and/or parallel elastic component of the vascular muscle tissues, which may explain some of the observed tension differences. If these plastic changes can fully explain the tension differences in Fig. 4 is uncertain. Firstly, all specimens were subjected initially to a tension of 200 gwt which was then not exceeded, in an attempt to eliminate further plastic changes from the start. Secondly, tension differences occurred on traction release of untreated muscle even in cases where the maximal tension was not greater than 50 gwt. The suggestion that normal vascular muscle has some 'catch' mechanism can probably not be excluded, therefore. Its eventually connection with the 'viscous resistance' of vascular smooth muscle is the subject of further studies.

Comparison between vascular and skeletal muscle

Certain fundamental biophysical properties are common to both vascular and skeletal muscle. Vascular, like skeletal muscle, is able to contract to 20–30% of its relaxed length. The length tension diagrams follow similar courses. The isometric tension per cm² cross section which can be produced maximally by a vascular muscle is of the same order of size as in skeletal muscle. Also, the energy required to produce certain tension is similar in the two types of muscle (Bevizi *et al.* 1965).

There are certain essential differences, however. Rigor mortis is not seen in vascular muscle on the disappearance of ATP. Series elastic components are 4–5 times greater in vascular than in skeletal muscle. The rate of contraction is about 100 times lower in vascular than in skeletal muscle. Normal arterial muscle is always more or less contracted and in an active state. On isometric contraction of a normal vascular muscle an increase occurs in the degree of active state but there is no change from a resting to an active phase, as in skeletal muscle. Terms such as 'resting length' and 'resting metabolism' strictly have no significance for vascular muscle.

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Reserpine and Gastrointestinal Mucosal Mast Cells¹

By

TOMMI RÄSÄNEN and EERO TASKINEN

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Abstract

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Rats were injected intra-abdominally with 2 mg/kg of reserpine once, 5 times and 9 times at 12-hr intervals. 4 hrs after the last injection the rats were killed and the mucosal mast cells of the stomach

Reserpine does not appear to cause changes in the peritoneal mast cells of the rat (Bhattacharya and Lewis 1956, Padawer 1957), though the 5-hydroxytryptamine content of the mast cells decreases (Moran and Westerholm 1963). Nor does reserpine cause morphologic changes in the mast cells of the subcutaneous connective tissue of the rat although their serotonin content especially declines (Parratt and West 1957).

The mast cell profiles of human gastric mucosa concur with the mucosal histamine and serotonin densities. The ratio between the concentrations of these amines in the human gastric mucosa is 13:1 (Murray Willis 1964), and in the rat 15:1 (Nikodijević and Trajkov 1963). The affinity of serotonin for heparin is considerably smaller than that of histamine (Selve 1965). For the rat skin the ratio between the histamine and serotonin concentrations is about 50, but in the wall of the gastrointestinal canal it varies from 4 to 8 (Talbot and East 1960).

Mast cell granules store and synthesise not only polysaccharides but also histamine and serotonin. The amount of serotonin decreases in the skin (Parratt and West 1957). Total release of serotonin happens in a mast cell population with a 10^{-4} M concentration of reserpine (Carlini, Fischer and Giarman 1964).

The mucosal serotonin content appears to remain unchanged in the gastric mucosa of the cat during reserpinisation whereas secretion increases (Emås 1964). As

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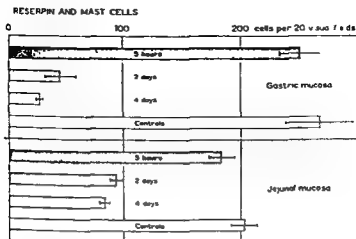


Fig. 1 The columns in the diagram indicate the count of mast cells per 20 visual fields in the gastric body and in the jejunal mucosa of rats

earlier studies have shown that rat gastric secretion during Shay ligature is affected by the different phases of the granulation of mucosal mast cells (Haikonen and Rasanen 1965), the authors decided to study the reactions of the mucosal mast cells during the reserpine treatment of rats

Method

Male rats of Dawley Sprague strain, aged about 5 months, were used in the study. They were kept in the laboratory on ordinary mixed rat diet and water ad libitum. The rats were injected with 2 mg/kg of reserpine (Serpassil, Ciba) once a day and 9 times at intervals of 12 hours. The injection was given intra abdominally.

The first group consisted of rats mean weight 332 g the second of 7 rats of which one died, mean weight 269 g and the third of 8 rats of which 3 died mean weight 221 g. The controls, 7 rats were given no injections. Their mean weight was 337 g.

4 hrs after the last injection the rats were bled under light ether narcosis. Immediately afterwards, samples were taken from the gastric and jejunal mucosa mounted on a small piece of cardboard and fixed in fresh 4% basic lead acetate. After fixation sections of 10 μ in thickness were cut vertically to the surface of the gastric mucosa and stained in 1% toluidine blue aqueous solution. The mast cell count was performed in the superficial part of the gastric body mucosa and the mean height of the jejunal mucosa from the zone richest in mast cells per 20 visual fields by means of oil immersion.

Results

Reserpine did not seem to cause changes in the gastric and duodenal mucosal mast cell amounts 4 hrs after its administration (Fig. 1). After 5 injections over a period more than 2 days (32 hrs) the number of gastric mucosal mast cells fell by 82% and



Fig 2

Fig 2 Loss of metachromatic material from the mucosal mast cells of gastric mucosa after four days reserpine treatment of rat. Magnification 300 \times



Fig 3

Fig 3 Submucosal connective tissue mast cells in the gastric wall of reserpine-treated rat. The mast cells contain profusely metachromatic granules. Magnification 800 \times

($P < 0.001$), decreasing further in the course of over 4 days (76 hrs) to 10 per cent of the control value ($P < 0.001$) (Fig 2). For the mast cells of jejunal mucosa the comparable decreases ranged from 50 to 60 per cent ($P < 0.001$).

Unlike the mucosal mast cells in the stomach, the mast cells of the submucosal connective tissue of the rats injected with reserpine displayed no features suggestive of degranulation even after 4 days (Fig 3). The rats were almost immobile after the reserpine injection and became increasingly exhausted as the reserpine treatment continued; they had no appetite, and one rat in the 2-day group and 3 in the 4-day group died. The stomachs of the rats given reserpine contained bloody gastric secretion, with the exception of one case in the 4-day group.

Discussion

Unlike the mast cells of the connective tissue (Parratt and West 1957) and peritoneal mast cells (Bhattacharya and Lewis 1956, Padawer 1957), in the gastric mucosal mast cells especially, but also in the mast cells of the jejunal mucosa, reserpine provokes degranulation. Gastric mucosal mast cells seem to be extremely prone to lose their metachromatic material under the action of glucocorticoids, ACTH, acute stress and irradiation (Räsänen 1961, 1963). It is possible that the amount of heparin mobilised in the urine during postoperative stress, which is four times the normal quantity (Mestel, Neuhof and Rosenfeld 1965), is mobilised chiefly from the liberal number of mast cells in the gastric mucosa.

Reserpine appears to be one of the most effective 5-hydroxytryptamine releasers in the central nervous system, the effect is, however, inhibited by monoamino-

enzyme inhibitors. Reserpine temporarily nullifies the ability of brain tissue to store serotonin. Gastric mucosa has an about 8—10 times higher serotonin concentration than the brain and the rat gastric mucosa is capable of storing a manifold quantity of exogenous serotonin without any changes in the histamine concentrations (Nikodym and Trajkov 1963).

The formation of endogenous serotonin in the gastric mucosa is probably a biological process as the turnover time of radioactive serotonin in the rabbit is short in the gastric mucosa (10 hrs), 17 hrs in the intestinal mucosa and 33 hrs in the spleen and in the platelets (Udenfriend and Weissbach 1958). The sharp increase in the serotonin concentration which occurs in portal blood under shock may be caused by the argentaffine granules of Kulitschitzky's cells or by platelets (Mark Hissen and Fellman 1964) and also by the serotonin which is released from mast cells of the gastrointestinal canal.

In addition to histamine, serotonin is probably released from the degranulated mast cells of the gastric mucosa under reserpine action. Serotonin has a greater affinity than histamine for heparin. Serotonin provokes mucosal lesions (Foley and Trajkov 1963), like reserpine, the ulcerative effect of the latter however is inhibited by monoamine-oxidase inhibitors or catecholamines (Decina and Krejci 1963).

Reserpine stimulates gastric secretion independently of the autonomous nervous system (Emlás 1964). Histamine disappears from the gastric mucosa in the course of the glucocorticoid effect when the mast cells of the gastric mucosa are degranulated (Foley and Glick 1962). Depending on the phase of granulation gastric secretion is stimulated or inhibited after the application of pyloric ligature in the rat (Haukonen and Rasanen 1965). Reserpine induced degranulation of gastric mucosal mast cells is probably accompanied by histamine liberation and parenchymal stimulation. Local liberation of serotonin may result in postcapillary spasm with mucosal hemorrhages.

Reserpine provoked degranulation of mucosal mast cells may be due to its direct effect or may be exercised by the hypophyseal — adrenocortical route which leads to the degranulation of mucosal mast cells (Rasanen 1961). In the rat, administered twice in a dosage of 10 mg/kg reserpine has a rather slightly increasing effect on the plasma cortisone content compared with ACTH. Consequently it is probable that the degranulation demonstrated in the present study is due to a great extent to the direct effect of reserpine on the mucosal mast cells.

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Cat Retinal Ganglion Cell Responses to Changing Light Intensities: Sinusoidal Modulation in the Time Domain

By

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Abstract

CLELAND, B and C ENROTH-CUGELL *Cat retinal ganglion cell responses to changing light intensities Sinusoidal modulation in the time domain* Acta physiol scand 1966 68 365—381

Action potentials were recorded from single axons of retinal ganglion cells in adult lightly anesthetized paralyzed cats (intact eye). A small sinusoidally time modulated stimulus was located in the most responsive portion of the receptive field center. The mean discharge frequency was monitored on a counter during the experiment and the responses over extended periods of time were recorded on tape and later averaged. Most of the cells were outside area centralis. A series of experiments showed that (a) the response amplitude was a function of modulation depth at constant frequency (b) a function of stimulus frequency at constant depth of modulation. Only two of 31 cells exhibited (up to 40 per cent modulation) a mean discharge frequency independent of modulation depth, a response amplitude reasonably proportional to modulation depth, an almost constant stimulus response phase difference and an averaged response whose shape was almost sinusoidal. The remaining cells failed to fulfill at least one of these requirements for linearity. For 6 cells modulation sensitivity curves were determined and found to have the same general shape as those subjectively determined in humans.

In a study of subjective flicker and fusion using sinusoidally time varying stimuli, deLange (1937) showed that under certain conditions the human visual system may be considered to operate linearly. The behavior was found to be linear under threshold conditions at fusion whereas deLange's experiments suggested that under conditions of marked flicker at low frequencies the response was definitely nonlinear. His work clearly demonstrated that linear filter theory — when applicable — is a useful tool in the study of visual systems. However, a linear approach to electrophysiologically recorded visual signals can be justified only if linearity of the visual processes involved is experimentally demonstrated. Linearity, if established und

threshold conditions in *psychophysical* experiments, does not permit conclusions regarding the nature of *neurophysiological* stimulus response relationships under threshold or suprathreshold conditions

Several investigators have employed sinusoidal stimuli in neurophysiological experiments on invertebrate and vertebrate eyes (Van der Tweel 1961, Gouras and Gunkel 1962, deVoe 1963, Kuiper and Leutcher-Hazelhoff 1965, Hughes and Maffei 1964, Maffei, Moruzzi and Rizzolatti 1965, Pinter 1966). Retinal action potentials were recorded by deVoe (1963) from the light adapted eye of the Wolf Spider in response to sinusoidally time varying stimuli. He found a mixture of both linear and nonlinear suprathreshold behavior, the response amplitude was proportional to the depth of modulation at constant frequency of stimulation and the stimulus response phase difference was independent of the depth of the modulation, as required for a linear system. However, the waveform of the response showed negligible deviation from a sinusoid at only low depths of modulation, above 10 per cent the waveform of the response showed definite nonlinearities. The response characteristics of cat retinal ganglion cells during stimulation with sinusoidally modulated lights have been briefly reported by Hughes and Maffei (1964), Maffei *et al* (1965) and Rackenspenger *et al* (1965).

The aim of the experiments presented here was to determine the degree of linearity of retinal signal generation and transmission and to observe the extent of non-linearity of the retinal ganglion cells. Although we realize that all parts of the receptive field contribute to the characteristics of the signal that travels in a retinal ganglion cell axon we elected first to study only responses from the receptive field center. By restricting ourselves to stimuli that involved the receptive field center only we became acutely aware of the very real problem of maintaining prolonged, effective immobilization of the eye. Without absolute stability of the globe it is impossible to obtain any quantitative measure of the steady state response to a small stimulus. We are reporting our experience with this problem as well as our findings regarding the response characteristics of cat retinal ganglion cells at supra threshold and at "threshold" levels during sinusoidal stimulation.

Methods

a. Preparation and recording

Adult anesthetized paralyzed and artificially respiration cats were used (For drug details see below).
 — Immobilization of the eye. The head was held in a stereotaxic instrument and a rectangular piece of bone (from Horsley Clarke F 10 to F 16 and LL 2 to LL 10) was removed from the top of the skull providing access to the chiasm and that part of the optic tract over which there is no lateral

b. *Stimulation*

For most experiments a low frequency light modulator (Gubisch 1965) was used to project a circular stimulus onto the middle of a rectangular white, cardboard screen placed one meter from the cat. The screen subtended 60×70 deg at the cat's eye and was evenly illuminated at 15 millilamberts. To obtain the desired luminance variation with respect to time the modulator was driven by a function generator in a sinusoidal or square wave fashion. The maximum mean luminance of the stimulus was 100 millilamberts. Its size could be changed without changing the mean luminance. The diameter of the smallest spot subtended 0.25 deg at the cat's eye. The modulation depth of the stimulus luminance was variable from zero to hundred per cent.

The stimulus spot was less than one deg in all the experiments. As a rule the receptive field centers were not mapped and the stimulus and center diameter can therefore not be compared in each case. However, the stimulus was always such that when placed centrally in the receptive field and modulated 100 per cent at 0.5 cps in a square wave fashion no audible impulse frequency increase could be detected at offset of light for on-center cells and vice versa for off-center cells.

In a few experiments a second stimulator was used. This consisted of six fluorescent tubes enclosed in a box behind opal glass located behind a central hole in a cardboard screen identical to the one above. The function generator was used to vary the light intensity. The light intensity sufficient to permit luminance modulation up to a maximum of 90 per cent. The stimulus diameter was varied by using cardboard screens with different sized apertures. The maximum mean luminance was 700 millilamberts which was decreased with neutral density filters inserted between the stimulus and the light source. The front of the screen was evenly illuminated at 1 millilambert.

Modulation depth m expressed in per cent is defined as $(L_{max} - L_{min}) / (L_{max} + L_{min})$ where L is the stimulus luminance. As mentioned above the stimulus spot provided by the first stimulator was superimposed on a non-modulated background of 15 millilamberts. It should be noted that all the numerical values given in the text and illustrations are corrected to give the true depth of modulation as observed by the cat (i.e. corrected for background). With the second stimulator this complication did not arise since in that case the stimulus spot was not superimposed on a non-modulated background. Stimulus and background strengths are given in terms of retinal illumination. With fully dilated pupil (14 mm diam) 1 millilambert corresponds to approximately 100 trolands with a 4 mm diameter artificial pupil approximately 40 trolands.

c. *Data Processing*

At some time following the experiment the tapes were played back and the data processed. The ganglion cell spikes were used to trigger a pulse shaper which gave a current pulse of 0.7 msec duration. The pulses were fed to a capacitor which discharged through a fixed resistor. The time constant of this RC smoothing network was 10 msec. The voltage across the capacitor was applied to one of the inputs of a digital memory oscilloscope (Enhancecon 1024) which formed the sum of a number of response cycles. This sum was scaled to give the average variation in impulse density of the retinal ganglion cell discharge. The stimulus was taken from the tape to the other Enhancecon input. A sweep duration equal to two stimulus cycles was chosen on the Enhancecon and generally 40 sweeps were averaged. Both averages were written out on an x-y plotter. Response amplitudes and phase differences were measured on the x-y record. Response amplitude refers to the peak peak amplitude of the impulse density fluctuations occurring in synchrony with the luminance modulation. For on-center cells the phase differences were measured from stimulus peak to response peak for off-center cells from stimulus trough to response peak (see Fig. 6 and 7).

Using an RC network for smoothing necessarily introduces a frequency dependent phase shift and attenuation. The actual amount of phase shift introduced by our equipment was experimentally determined at 4, 8 and 16 cps and found to be 15, 27 and 30 deg respectively.

Results

1. *Immobilization of the eye*

To collect data for curves relating the mean discharge frequency of the cell and the response amplitude to the depth of modulation or for plots of response amplitude versus frequency of stimulation at least two hours of unflinching immobilization of the eye was needed. Each time the depth of modulation or the frequency of the stimulus was changed the response from the cell was allowed to settle down to a new steady value before impulse train samples were recorded on the tape. During the ear

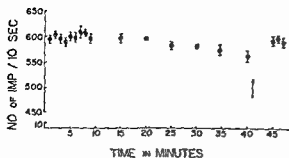


Fig 1 Mean discharge rate of an on center cell responding to a stimulus modulated 50 per cent at 8 cps. The stimulus which subtended 0.5 deg at the cat's eye was

the average count over one preceding minute. The length of the vertical bars is two standard deviations. The position of the stimulus was adjusted at the arrow.

doses commonly used by others in similar experiments. Mostly doses below 15 mg/kg/hr of succinylcholine chloride or gallamine triethiodide have been used (Wiesel 1960, Bishop, Kozak and Vakkur 1962, Burns and Pritchard 1964, Kozak, Rodieck and Bishop 1964, Levick and Williams 1964, Ogawa, Bishop and Levick 1966). In one case (Rodieck and Stone 1965a) 20–25 mg/kg/hr of gallamine was reported. The paralysis that we achieved with such dose rates was far from satisfactory for our purposes. We were interested in quantitative aspects of the steady state output from the ganglion cells in response to a small constant stimulus. Whether the quantity measured is the mean spike frequency or the response amplitude, results are reliable only if one can trust that observed changes in the magnitude of the output are due to changing the frequency and/or depth of modulation of the stimulus, not to a relative image motion within the receptive field. To learn how to achieve long lasting eye immobilization in the face of light anesthesia a series of stability experiments were conducted on 52 cells (22 on center and 30 off center cells) in 17 cats. Light anesthesia is highly desirable in order to minimize the effect of the anesthetic upon the retinal function (Barlow, Hill and Levick 1964).

The procedure was as follows: when the receptive field had been located the stimulus size was decreased to the minimum at which a good response was heard. By moving the stimulus around within the receptive field center the position which yielded the highest mean impulse frequency (as noted on the electronic counter) was selected and the stimulus was left in that position. When after 1st–30 sec the count had seemingly settled down to a steady state the number of impulses per 10 sec interval was observed and plotted versus time.

Fig 1 illustrates the effect upon the mean spike frequency of an on center cell of a slow drift of the eye. The stimulus diameter subtended 0.5 deg at the cat's eye. During the first 1.5 min the output from this ganglion cell was quite steady but then a slow decline of the mean frequency started. At 40 min the counting was interrupted and the stimulus was moved to various locations within the receptive field center in search for one that yielded a higher count. In this particular case a 0.5 deg movement nasally brought the mean frequency very close to the level at which it had been 30 min earlier. Note that the instability in the impulse count illustrated in Fig 1 occurred while the gallamine triethiodide dose was 40 mg/kg/hr.

When such slow changes in the impulse counts occurred it was possible (with one exception, see below) to bring back the count to its original value by careful readjustment of the image within the receptive field. This suggested to us that the changes in the mean discharge frequency of the response to a constant stimulus were due to slow drifts of the eyes. The stimulus movements required varied from a few minutes of arc to more than 1 deg in the extreme cases. It might be argued that the extended exposure of a particular region of the receptive field to the sinusoidally modulated stimulus resulted in a 'local fatigue' and that when the stimulus was moved, involvement of a 'fresh' receptive field area resulted in increased impulse frequency. We do not believe that this is so because (1) An increased dose rate of the paralyzing agent often turned a spike count of the type shown in Fig. 1 into an hour long stable mean discharge frequency. (2) If adjustments as large as 1 deg or more of the stimulus position increased the impulse count, because a new 'non fatigued' area of the receptive field got involved, and not because the stimulus was brought back to the middle of the center, then one would have to assume that there were two rather widely separated locations within the receptive field which both responded maximally to the identical restricted sinusoidal stimulus. This is highly unlikely in view of what at present is known about the sensitivity distribution within the receptive fields of cat retinal ganglion cells (Kuffler 1953, Rodieck and Stone 1965b, Enroth Cugell and Robson 1966).¹

One experiment constituted an exception to the rule that slowly decreasing spike counts could be restored by moving the stimulus. This was done with a cell from a cat. For all

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In our earliest experiments in this series thiopentol or pentobarbitone sodium were combined with continuous intravenous infusion of succinylcholine chloride. When the narcosis was light (as judged by lively retinal reactions to stimuli throughout the experiment and slight general motor unrest upon removal of paralysis) good eye immobilization was not obtained until the succinylcholine doses reached such levels that many cats died early in the day of what clinically appeared as lung edema. Much better general condition of the animal was achieved with thiopentol or pentobarbitone in conjunction with gallamine triethiodide in high doses (up to 60 mg/kg hr). However, the pronounced effects of even small variations in barbiturate doses upon retinal activity (e.g. Barlow *et al.* 1964) were quite disturbing. Moreover, pentobarbitone particularly resulted in an annoying 'spontaneous' rhythmicity of the ganglion cell discharge as described in rats by Brown and Rojas (1965). In later experiments barbiturates were therefore avoided for maintenance of anesthesia and urethane chloralose was used (Anesthesia was induced with diethyl ether followed by about 5 mg/kg of thiamyl sodium intravenously). In view of Berglund, Nälén and Wallentin's (1965) findings on cats that the combination of urethane and chloralose is particularly apt to result in acidosis, the chloralose was abandoned and urethane

¹ See note 1 added in proof.

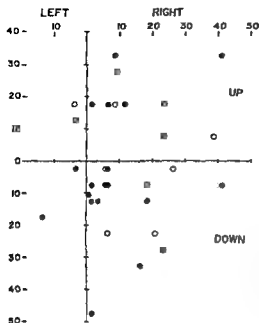


Fig. 2. Approximate positions in the visual field of receptive field projections. Open circles on center cells; filled circles off-center cells. The axes are marked in angular distance (deg) from the projection of the center of the area centralis.

alone used. Light anesthesia was maintained with doses of 3–5 mg/kg/hr. Before administration of gallamine, atropine and phenylephrine slight motor unrest was present and the pupils reacted well to light, directly and indirectly. The retinal responses were brisk throughout the experiment and when after 8–10 hours the gallamine infusion was discontinued spontaneous breathing and some motor unrest returned. In the cases where no atropine or phenylephrine had been instilled the pupillary reactions to light were normal.

Our results from this series of experiments may be summarized as follows: if a stimulus, which is smaller than the receptive field center, is modulated sinusoidally about a constant mean luminance at a constant frequency and at a constant depth the resulting mean spike frequency stays quite stable (coefficient of variation $\pm 3\%$) provided no eye movements occur and the preparation is in good general condition. Unless one resorts to deep anesthesia, it is our experience that doses of 20–60 mg/kg/hr gallamine triethiodide are needed to achieve reliable long lasting immobilization of the eyes. These doses are much larger than usually reported. High succinylcholine chloride doses seem to be less well tolerated than high gallamine triethiodide doses.

Response characteristics of retinal ganglion cells to stimuli of different modulation depths and frequencies

In a second series of experiments the response characteristics of retinal ganglion cells were studied in ten cats (13 on-center and 18 off-center cells). The position of the projection in space of the receptive fields of these cells relative to the projection of area centralis is shown in Fig. 2. The angles were estimated to within 5 deg and cor-

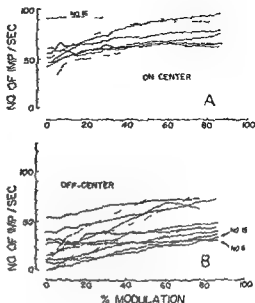


Fig 3 Mean discharge frequency of 8 on center (A) and 12 off center (B) cells in response to sinusoidally modulated stimuli at 8 cps. The depth of modulation varied. Full lines stimulus mean 5×10^4 trolands background 7.5×10^4 trolands. Broken lines stimulus mean 6×10^4 trolands background 5×10^4 trolands. The stimulus was smaller than the receptive field center and located in its most sensitive part.

rected for the direction of the optic axes in cats paralyzed with succinylcholine chloride (Vakkur, Kozak and Bishop 1963). Clearly only a minor portion of our units were central ones.

As in the previous set of experiments a small sinusoidally time modulated stimulus was positioned in the most responsive part of the receptive field center. Two main types of experiments were performed: (a) the frequency of the luminance modulation was held constant while the depth of modulation was varied; (b) the depth of modulation was constant, the frequency of modulation was varied. The mean discharge frequency of the cell, the response amplitude and the stimulus response phase difference were studied in (a) as a function of modulation depth and in (b) as a function of modulation frequency. In a third type of test the experimenter determined the depth of modulation required at different frequencies to evoke a barely discernable response to the sinusoidal stimulus, i.e., threshold curves similar to deLange's (1937) attenuation characteristics of the human visual system were determined for a few retinal ganglion cells.

a. Constant frequency of modulation

The output of a linear system in response to a sinusoidal input of constant frequency is characterized by: (1) a mean value that is independent of the modulation depth of the input; (2) a stimulus response phase difference independent of variations of input modulation; (3) a response amplitude which is proportional to the depth of the input modulation; and (4) a waveform which is a replica of the sinusoidal input. To assess the extent of linear and nonlinear features of retinal ganglion cell response

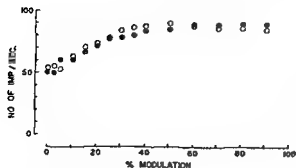


Fig 4 Two determinations of mean impulse frequency as a function of modulation depth for the same on center cell. The stimulus frequency was 8 cps. Open circles the first determination. Filled circles the second determination which started approximately 15 min after the first one was completed.

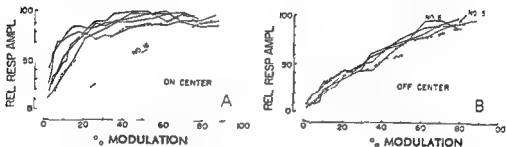


Fig 5 Relative response amplitude as a function of modulation depth. Stimulus frequency 8 cps. A on center cells B off center cells. Full lines stimulus mean 5×10^4 trolands background 7.5×10^4 trolands. Broken lines stimulus mean 6×10^4 trolands background 5×10^4 trolands. The stimulus was smaller than the receptive field center and located in its most sensitive part.

the four above mentioned characteristics of the cell discharges were studied during constant frequency stimulation and the results are reported below. In the majority of these experiments the stimulus frequency was 8 cps. This particular frequency was selected because our preliminary experiments with sinusoidal stimuli showed that the retinal ganglion cells were most sensitive at stimulus frequencies around 8 cps. This frequency falls within the region of maximum sensitivity of deLange's human attenuation curves (1957) as well as within the range of maximal mean discharge frequency for cat retinal ganglion cells stimulated with diffuse light modulated at 100 per cent in a square wave fashion (Grusser and Reidemeister 1959).

Mean spike frequency. In Fig 3 the mean spike frequency recorded from 8 on center and 12 off center cells is plotted as a function of modulation depth. The experimental procedure was to start with the deepest modulation and then decrease it in small steps. For each new depth of modulation the number of pulses per 10 sec interval was observed on the electronic counter until the value was stable. The average of three 10 sec counts was then noted. (The response was also recorded on tape and later processed to yield the response amplitude for each depth of modulation.) When the spike count for zero per cent had been obtained the modulation was again set at the highest value and the entire procedure repeated. The two curves in Fig 4 each represent one full run from highest to lowest depth at modulation. This is a

representative sample of the repeatability (provided eye immobilization is reliable) of results obtained in the type of experiments where mean discharge frequency and/or response amplitude was studied as a function of modulation depth and/or modulation frequency. By checking in random order a few points on the curves in Fig. 3—5 and 9 it was established that their shape was not influenced by the experimental procedure. Eight of the curves in Fig. 3 represent a single run from highest to zero per cent modulation, the others are averages of two or three runs. Note that only one on center cell (no. 16) and one off center cell (no. 6) maintained a constant mean discharge frequency with changing modulation depth, at a constant frequency of stimulation. In the case of all the other cells the mean discharge rate depended upon the depth of modulation, although not in the same fashion for all cells.

For some cells data regarding the mean discharge frequency of the cell as a function of modulation depth were obtained at more than one constant stimulus frequency. The on center cell (no. 16) whose stable mean discharge rate during an 8 cps stimulus is shown in Fig. 3 A, was also tested at 4 and 15 cps, both these curves were as "flat" as the 8 cps curve. The off center cell (no. 6) with constant mean discharge frequency at a stimulus frequency of 8 cps was not tested at any other frequencies. A total of 7 cells (3 on, 4 off center cells) with sloping mean discharge frequency curves were stimulated at several constant frequencies of modulation (ranging from 0.5—28 cps). The mean discharge rate rose with increasing modulation depth at all frequencies which yielded a measurable response amplitude when the data were averaged, i.e. when the cell responded with an impulse frequency modulation.

All the curves in Fig. 3 were obtained with much the same retinal illumination (5×10^4 or 6×10^4 trolands). It is reasonable to question the effect of different mean values of the stimulus luminance upon the average discharge rate of the cell as a function of modulation depth. Unfortunately, we have very little experimental results at varying retinal illumination levels of the stimulus. However, two on center and two off center cells were studied (at 8 cps) at 5×10^4 and 5×10^5 trolands while the background was 5×10^4 trolands. Only with stimuli of the higher mean retinal illumination was there a significant rise in the mean discharge frequency of these cells as the modulation depth increased. Possibly the manner in which the mean discharge rate of retinal ganglion cells depends upon the modulation depth of a constant frequency stimulus, is influenced by the mean retinal illumination of the stimulus.

Relative response amplitude. Fig. 3 shows relative response amplitude at 8 cps as a function of modulation depth for seven of the on center cells and seven of the off center cells of Fig. 3. The magnitude plotted is the peak peak response amplitude as measured on the x-y record (see Fig. 11 and Fig. 7, second trace from above). The curves have been normalized for 100 per cent. The response amplitude of on center cells increase much more rapidly at low depth of modulation than do off-center amplitudes. The on-center curves, with one exception, begin to

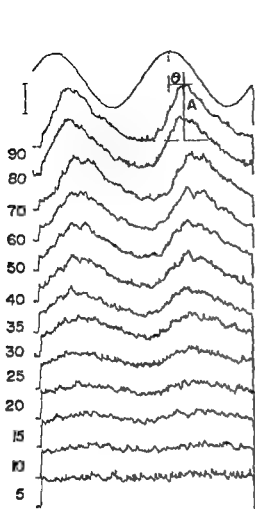


Fig 6

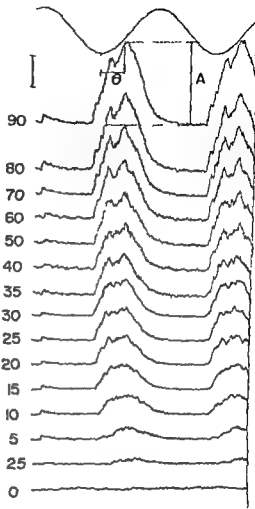


Fig 7

Fig 6 Averaged impulse discharge from one on-center cell (No. 16) in response to sinusoidally modulated stimuli at 8 cps. The top trace indicates the stimulus; the others are the output from the cell at varying depths of stimulus modulation. The number to the left of each trace is the per cent modulation. The height of the vertical bar in the upper left corner corresponds to 100 imp/sec. Zero imp/sec is indicated by the initial short horizontal portion of each trace. The angle θ between the stimulus peak and the response peak is the phase difference. The distance Δ between the minimum and the maximum impulse frequency during each cycle is the response amplitude. Note that although the response amplitude increases with increasing modulation depth, the mean discharge frequency for this cell is approximately 90 imp/sec in all tracings. Stimulus mean 6×10^4 trolands; background 5×10^3 trolands.

Fig 7 Averaged impulse discharge from an off-center cell responding to sinusoidally modulated stimuli. The top trace indicates the 8 cps stimulus; the other traces are the output from the cell

between the minimum and the maximum impulse frequency during each cycle is the response amplitude. Stimulus mean 6×10^4 trolands; background 5×10^3 trolands.

approximately 20 per cent while the off-center curves tend to continue to rise with rather little change of slope far beyond 20 per cent modulation. The response amplitude curve of the on-center cell (Fig. 3A) which exhibited a flat mean discharge frequency versus modulation curve (no. 16) is of a rather different character than its fellow curves: for this one on-center cell the amplitude of the response increased in a nearly linear fashion (at 8 cps) with increasing depth of modulation.

Waveform of the response. In Fig. 6 and 7 are shown the averaged responses from one on- and one off-center cell during stimulation at constant frequency but changing depth of modulation. In the case of this on-center cell the waveform is approximately sinusoidal up to 30–35 per cent modulation. This is the same on-center cell (no. 16) whose mean impulse frequency was independent of depth of modulation (Fig. 3A), and whose relative response amplitude at 8 cps was almost linearly related to the modulation depth (Fig. 5A).

For the off-center cell shown in Fig. 7 (at 8 cps) the waveform of the response is clearly non-sinusoidal even at the lowest depths of modulation. Not only does the response appear rectified at all depths but at higher modulation levels there is in addition a double peak. This was not the case for all off-center cells. The mean spike frequency and the response amplitude versus modulation curves marked no. 15 in Figs. 3B and 5B are from an off-center cell where the waveform of the impulse frequency modulation (at 8 cps) was an almost perfect sinusoid up to 40 per cent modulation. However, the off-center cell responses tended to exhibit more pronounced deviations from sinusoidal shape at lower levels of modulation than did the on-center cells. Under our stimulus conditions gross distortion of the waveform became apparent at modulation depths varying from 35 to 70 per cent for seven on-center cells while the corresponding figures for seven off-center cells was 25 to 40 per cent.

Stimulus response phase relations. The phase difference between stimulus and response was measured as described in Methods and as indicated in Fig. 6 and 7 for on- and off-center cells respectively. At 8 cps and deep modulation both types of cell exhibited an approximately 70 deg phase lag (Fig. 8). As the depth of the modulation decreased the phase lag increased, more so for on-center cells than for off-center cells. The phase shift introduced by the data processing system at 8 cps amounts to 27 deg (see p. 367). The phase angles plotted in Fig. 8 include this phase shift contributed by the data processing.

b. Constant depth of modulation

In this section some characteristics of retinal ganglion cell responses as a function of frequency will be described. As before, the stimulus was located in the most responsive part of the receptive field center. The experimental procedure for obtaining curves, which relate mean impulse frequency, response amplitude and stimulus response phase difference to stimulus frequency was similar to the one used in the previous section. The only difference was that in these experiments the stimulus parameter being varied was the frequency of modulation, not its depth.

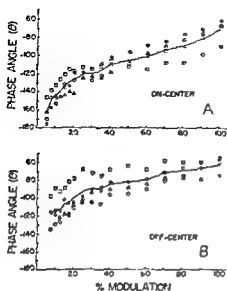


Fig 8

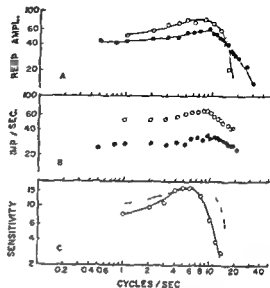


Fig 9

Fig 8 Graph of phase shift θ (see Figs 6 and 7) as a function of stimulus modulation depth (stimulus frequency 8 cps) A four off center cells B four on-center cells Different symbols represent

scaling is arbitrary

B mean impulse frequency for the on center (open circles) and the off center (filled circles) cell in A Modulation depth 50 per cent

C modulation sensitivity curve for the same on center cell as in A and B For each frequency the experimenter determined the depth of modulation at which he could barely hear an impulse frequency modulation synchronous with the luminance fluctuations The inverse of these values are plotted (open circles) The broken line is the frequency response curve for the same cell (shown in A)

Stimulus mean in A B and C. 5×10^4 trolands background 7.5×10^3 trolands

Mean spike frequency and response amplitude Frequency response curves were successfully completed for 10 ganglion cells (four on- and six off-center cells) The stimulus mean was 5×10^4 trolands (background 5×10^3 trolands) except for one on- and one off-center cell where it was 4×10^3 trolands (background 40 trolands) All the frequency response curves obtained at modulation depths higher than 15 per cent exhibited one frequency at which the response was at a maximum, falling off both at lower and at higher frequencies Similarly the mean discharge rate of the cell reached a maximum at some intermediate frequency of stimulation Fig 9 shows representative frequency characteristics for one on- and one off-center cell A and B are plots of the response amplitudes and the average impulse discharge frequencies at 50 per cent modulation. The curve in Fig 9C represents an auditory determination of the smallest modulation depth required at each individual frequency for the experimenter to barely detect an impulse frequency fluctuation synchronous with the stimulus modulation The values plotted are the inverse of this "threshold"

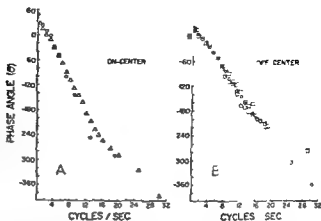


Fig 10 Graph of phase shift θ (see Fig 6 and 7) as a function of stimulus frequency. Depth of modulation 50 per cent. A two on-center cells. B three off-center cells. Stimulus mean 5×10^4 trolands background 7×10^6 trolands.

modulation. No such sensitivity curve was determined for the off center cell shown in Fig 11. Modulation sensitivity curves were determined for three on- and two off-center cells and they all had the general character of the curve shown in Fig 11, i.e. a maximum at some intermediate frequency of stimulation.

Stimulus response phase relationships. Fig 10 shows representative plots of the phase shift as a function of stimulus frequency for two on- and three off-center cells. The modulation depth was 50 per cent. The plots have not been corrected for the phase shift introduced by the averaging procedure (see p. 367). It can be seen that at the very lowest frequencies there is a phase lead, i.e. the on-center response reaches its peak prior to the luminance maximum, the off-center response prior to the luminance minimum. (Correction for the error due to the averaging equipment would increase the amount by which the response leads at these low frequencies.) As the stimulus frequency increases the phase lead turns into a steadily increasing phase lag, the increase being more pronounced for the on- than for the off-center cells.

Discussion

1. Immobilization of the eye. We have found the dose rates of muscle relaxants commonly used in experiments on cat retinal ganglion cells far too small for our purposes. The majority of our receptive fields were in the peripheral retina (see Fig. 2) and therefore most likely had field centers whose diameters were 2 deg or more. Wiesel (1960, Enroth-Cugell and Robson 1966). Thus, as a rule our stimulus diameter (less than 1 deg) was small compared to the diameter of the receptive field center. Considering that the sensitivity falls off quite rapidly with distance from the central point of maximum sensitivity (Kuffler 1953, Rodieck and Stone 1963b, Enroth-Cugell and Robson, 1966) one expects the steady state output of the cell in response to a restricted stimulus to be critically dependent upon the position of the stimulus within the receptive field center. It is possible that other workers have had less need for longlasting perfect immobilization of the eye because (1) they have not been interested in quantitative aspects of the steady state response of the cell or (2) they have been studying the steady state behavior but used a stimulus much larger

than ours compared to the diameter of the receptive field center. Ogawa *et al* (1966) studied steady state responses from cat retinal ganglion cells with receptive field centers smaller than ours (all their recordings were from area centralis) while maintaining muscle paralysis with 8 mg/kg/hr of gallamine triethiodide. Yet their smallest stimulus was 1.4 deg. Possibly the mechanical support of the eye employed by these authors was partially responsible for their findings that "there was usually no apparent change in the discharge characteristics of the ganglion cells over the long periods (more than 4 hrs) that were required to complete the detailed study of each unit". On the other hand, they also state "under apparently steady state conditions both the spontaneous and the evoked discharges would occasionally show temporary alterations in the mean firing rate". Perhaps these alterations were caused by eye motions similar to those we believe responsible for the slow fluctuations in mean frequency of discharge that were so disturbing in our experiments.

The gallamine triethiodide dose rates with which the majority of the results reported here were obtained are three to five times larger than those reported by others. One may question if such large doses possibly have an effect upon the central nervous system in general and on retinal function in particular. We have noted no retinal ganglion cell behavior that we suspect is related to the gallamine administration nor have we found any indication in the literature that such effects could be expected.

2. Response characteristics. In our study of retinal ganglion cell response characteristics during stimulation with sinusoidally modulated lights we focused our attention on four aspects of the stimulus-response relationship: mean discharge frequency, relative response amplitude, the waveform of the response and the phase shift. Among a total of 31 cells we found only two cells (no. 16 and 6) which, at constant frequency of stimulation (8 cps), behaved sensibly linearly up to approximately 40 per cent modulation in the following 3 respects: (1) the mean discharge frequency of the cell did not depend upon the modulation depth, (2) the response amplitude was reasonably proportional to the depth of the stimulus modulation, (3) the waveform of the response was almost sinusoidal. However, the claim that these two cells (over a limited range of modulation depths, at the one mean luminance level that we used) in fact operated linearly requires their stimulus response phase-shift to be independent of modulation depth (to 40 per cent). For the on-center cell (no. 16) this was true, but for the off-center cell it was not. Among all the off-center cells this particular one (no. 6) exhibited the least increase in phase shift at low modulations (see square symbols Fig. 8B). Thus within the restricted conditions of our experiments, only these two cells appeared to behave reasonably linearly but it should be remembered that we did not use different mean luminances nor did we do any superposition experiments.

The remaining 29 cells behaved somewhat differently. In general terms, if one of the 4 aspects of the output that we studied showed a relatively linear relationship to the stimulus, one or more other aspects did not. For example, all the off-center curves relating response amplitude to depth of modulation in Fig. 5B are tolerably linear

up to high modulation depths. Yet the mean discharge frequency of all these cells (except no. 6) depended upon the modulation depth and for some of the cells the waveform was grossly distorted even at the very lowest modulation depths.

Our cell sample is not large, does not include many cells from the central retina and our experimental data are incomplete in some aspects. In spite of these shortcomings our results contradict such generalizations as that the retinal ganglion cell discharge can be modulated in an almost linear fashion by photic sine wave stimuli (Hughes and Maffei quoted from Maffei *et al.* 1965). However Hughes and Maffei used diffuse retinal illumination and no details of their results are available. Rackensperger *et al.* (1965) stimulated the receptive field center and their very short preliminary report does not indicate linear retinal ganglion cell behavior. The results presented here are in agreement with those of Enroth Cugell and Robson (1966). They concluded that photoreception, initial signal transmission and summation is as linear for some cat retinal ganglion cells but they made no claim that the response of a ganglion cell is linearly related to the stimulus magnitude. It is interesting to note that although we in general found retinal ganglion cell responses to be nonlinear we did not note any threshold type nonlinearities although some cells were tested at modulation depths as low as 0.25 per cent. This agrees well with the findings of Enroth Cugell and Robson (1966) in experiments with moving sinusoidal grating patterns.

In spite of variations between individual cells of on- and off-center type there was a trend to consistent differences between the two groups. The mean discharge frequency at 0 per cent modulation was on an average higher for on-center cells (as must be expected with a stimulus located in the receptive field center (cf. Fig. 3A and 3B)). A saturation type nonlinearity was typical for on-center cells but not for off-center cells in the curves relating response amplitude to modulation depth at a constant frequency of stimulus. There was a greater decrease in phase lag with increase in stimulus modulation depth as well as a more pronounced increase with rising stimulus frequency for the on-center cells than for the off-center cells. Marked deviation of the waveform of the response from a sinusoid mostly took the form of rectification in the case of off-center cells; double peaks were also observed. For on-center cells the distortion was more often of the type seen in Fig. 6 at the highest depths of modulation occasionally in conjunction with clipping.

The frequency characteristics of both on- and off-center responses (suprathreshold conditions) all had the same general shape: a maximum at an intermediate frequency of stimulation falling off towards both lower and higher frequencies. The modulation sensitivity curves (i.e. frequency characteristics at threshold) were all obtained with an auditory method. Properly such curves should be objectively determined: response samples should be recorded for each stimulus frequency at several depths of modulation. From the averaged data the modulation depth which at each frequency results in a constant small response could then be found. Such procedure is however too time consuming to be realistic. Sensitivity curves for auditory type are clear.

ganglion cell involved but also an auditory threshold of the experimenter. Further it is not unlikely that the criterion chosen by the experimenter varies with stimulus frequency and with the average discharge frequency of the cell. On the other hand, for any individual cell such modulation sensitivity curves can be repeated quite satisfactorily after a period of training. All the modulation sensitivity curves that we determined with the auditory method were of the same general shape as the frequency characteristics of retinal ganglion cell responses. They were also of the same general type as deLange's (1957) subjectively determined human modulation sensitivity curves. From our experiments it would thus appear that there is an analogy between psychophysical results obtained in humans with sinusoidal stimuli at threshold and the frequency response characteristics of retinal ganglion cells in the cat, both at 'threshold' and at suprathreshold level. This suggests that the frequency response characteristics of the human visual system are at least partly determined at the retinal level, provided we are justified in arguing from the cat to the human visual system.

Notes added in proof

amplitude-modulation curves to yield suprathreshold plots of modulation sensitivity versus stimulus frequency at two different constant response levels. These sensitivity curves had the same general shape as the modulation sensitivity curve at threshold (Fig. 9C) and the response amplitude curves (Fig. 9A).

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Circulatory and Respiratory Adaptation during Prolonged Exercise in the Supine Position

By

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Abstract

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Circulatory and respiratory changes during prolonged non steady state exercise in the supine position were studied by the heart catheterization technique in six healthy young men of average physical fitness. The work load was chosen so that they could perform supine exercise for one hour. This load gave a mean heart rate of about 128 beats/min after 10 min and 142 beats/min after 50 min of work. The circulatory changes during these experimental conditions were characterized

characterized by a slight increase in total ventilation, an unchanged alveolar ventilation and a slight increase in dead-space ventilation. The small increase in total ventilation was due to a small increase in respiratory rate and tidal volume. The overall ventilation-perfusion relationship was unchanged. Both circulatory and respiratory changes may be explained by a decreased tone in the capacity vessels with a shift in the distribution of the blood.

In an earlier study (Ekelund and Holmgren, 1964) the adaptation of the circulation and respiration was studied during non-steady-state exercise for one hour in the sitting position. During these experimental conditions there was a continuous fall in the stroke volume and systemic arterial blood pressure, but the cardiac output was maintained constant by an increase in heart rate. The respiratory response was characterized by an increased total ventilation, with a moderate increase in alveolar ventilation and a marked increase in dead-space ventilation. The overall ventilation-perfusion ratio of the lungs showed a slight continuous increase. The circulatory and respiratory changes might be explained by a gravitational shift in the blood volume due to a decrease in the tone of the capacity vessels. If so, the possibility of maintaining a steady state should be better in the supine than in the sitting position, when the influence of gravitation should be diminished.

A preliminary report was given at the Meeting of the Swedish Society for Clinical Physiology, Stockholm, Nov. 1963.

In a metabolic study on a similar material Carlson *et al* (1964) however found a fairly marked increase in heart rate during exercise for 60 min in the supine position. The present investigation was therefore planned to study whether stroke volume behaves in the same way in supine as in sitting position under similar experimental conditions and with equivalent material.

The circulatory response was studied by the heart catheterization technique and the respiratory changes by means of arterial blood and expired air samples.

Material

The subjects consisted of 6 healthy male volunteers aged 20–28 years. They were all in normal health prior to the investigation. The investigation was approved by the local ECG at

Methods

The subjects were
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Right heart catheterization was performed by the conventional technique from the left arm with a No 9 double-lumen catheter. The right brachial artery was catheterized percutaneously. Blood pressures were recorded with an electrical transducer and with the reference point at the mid thoracic level. The cardiac output was measured by the direct Fick method. Expired air was collected in Douglas bags and was analysed for oxygen and carbon dioxide by the Haldane technique. The heart rate was determined from an electrocardiogram.

The respiratory
saturation and
Pernow 1959).

PCO₂-electrode

Two cases PCO₂ was determined by the Astrup technique, to which values 3.0 mm Hg was added (Ekelund and Holmgren 1964, Holmgren 1965). The pH standard bicarbonate and base excess were determined according to Andersen (1963). Measurements of pH and PCO₂ were corrected for differences between body temperature and electrode temperature. Lactic acid concentration was determined by the spectrophotometric method of Barker and Summerson.

The statistical calculations were performed according to Snedecor (1959).

Procedure

Prior to the experiment a pilot test was performed to choose a load that the subjects could sustain for one hour in supine position. Because of the difficulty of exercising in supine position these loads were somewhat lower than those used in the earlier study in the sitting position (Ekelund and Holmgren 1964). Expressed in per cent of their working capacity at pulse 170 (W₁₇₀) the subjects worked at a level of 59.7 (57–63) % in supine against 77 (67–85) % in the sitting position.

The experiment was

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brachial arteries card

bonate were measured.

in the same body position and continued for one hour at the load selected in the pilot test. The pedal axis of the bicycle was located 20–24 cm above the table with a stroke radius of 18 cm. Every 10th minute expired air was collected for 3 min while blood samples were withdrawn.

TABLE 1 Anthropometric data in 6 healthy young men. The symbols are the same as in Fig. 1-4

Case no	Age, years	Height, cm	Weight, kg	Heart, vol, ml	THb, g	Hb, konc, g/100 ml	Blood, vol., l	W_{170} , kpm/min sitting	W_{170} , kpm/min supine
1	23	192	86	960	985	13.8	7.80	1,250	1,400
2	28	191	79	965	800	13.4	6.60	1,100	1,150
3	21	186	83	915	915	14.7	6.80	1,150	1,200
4	24	180	72	800	700	13.8	5.60	1,000	1,000
5	24	174	68	650	560	12.3	5.05	1,000	900
6	20	187	69	740	700	12.3	6.25	1,150	1,200

the brachial and pulmonary arteries for the determination of oxygen saturation, tension and content, hemoglobin concentration, carbon dioxide tension, pH, standard bicarbonate, base excess and lactate concentration. After 10 and 40-50 min of work [125 I-labelled albumin was injected for blood volume determination. Rectal temperatures, ECG, heart rate, brachial arterial, pulmonary arterial and right ventricular pressures were monitored continuously. In 3 subjects the pulmonary arterial wedge pressure was measured after 10 and 60 min work. The work test was continued for 60 min in 5 subjects. No complications occurred before, during or after the test. The blood loss was of the order of 150 ml. The total fluid losses (blood and sweat) were approximately compensated for by continuous saline administration. The room temperature was between 21 and 23°C with a relative humidity of about 40%.

Results

Anthropometric data are given in Table 1.

The total amount of hemoglobin (THb) was on an average 778 g (range 760-985) corresponding to 10.1 g per kg b.w. (range 9.8-11.6), which is about 10 per cent lower than earlier values reported from this laboratory (Holmgren *et al.* 1960). This difference is due to a difference in absolute calibration of the carbon monoxide reference gas. The mean value for the hemoglobin concentration was 13.4 per 100 ml blood (range 12.3-14.7), which is of the same magnitude as in the above-mentioned material.

The total blood volume determined by the alveolar carbon monoxide method was on an average 6.35 l (range 5.05-7.80), or 83 l/ml (range 74.3-90.7) per kg b.w.

The total blood volume, determined with [125 I-labelled albumin, was on an average 5.88 (range 5.0-7.0) l in 5 subjects. The CO blood volume for the same individuals was on an average 6.06 l. After 10 min work the blood volume had decreased to an average of 5.62 l, a decrease of 260 ml or 4.4 per cent of the initial value (not significant). After 50-60 min work the average blood volume was 5.54 l, which is not significantly different from the value after 10 min work. The corresponding plasma volumes were 3.64 l at rest, 3.40 l after 10 min work, and 3.38 l after 50-60 min work.

The heart volume in the prone position averaged 838 ml (range 650-965).

The rate of work that could be performed at the heart rate of 170 beats per min (W_{170}) was on an average 1063 kpm/min (range 1000-1250) in the sitting position. There was no significant difference between W_{170} in the supine and sitting position (average 1138 kpm/min in supine).

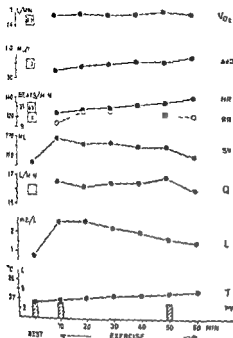


Fig. 1

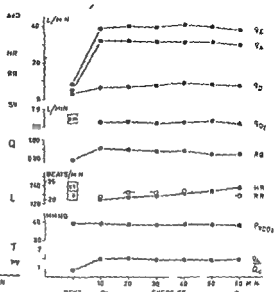


Fig. 2

Fig. 1 Hemodynamic data at rest, data in squares, and during exercise for one hour in six subjects V_{O_2} = oxygen uptake, l STPD/min AVD = arteriovenous oxygen difference ml/l HR = heart rate beats per min, RR = respiratory rate breaths per min SV = stroke volume ml Q = cardiac output, l/min, L = lactic acid in arterial blood, meq/l, T = rectal temperature C_{PV} = plasma volume, l

Fig. 2 Respiratory data at rest and during exercise in 6 subjects V_E = total ventilation l BTPS/min, V_A = alveolar ventilation, l BTPS/min V_D = deadspace ventilation, l BTPS/min V_{O_2} = oxygen uptake l STPD/min, RQ = respiratory quotient HR = heart rate beats/min RR = respiratory rate breaths/min, P_{aCO_2} = arterial carbon-dioxide tension mm Hg V_A/Q_c = ventilation perfusion relationship

The anthropometric data indicate that this material did not differ significantly from the material used for the study in the sitting position (Ekelund and Holmgren 1951) with the exception of the pulse rate in standing position which was on an average 88.7 beats/min in this material and 77.8 beats/min in the earlier with the same resting pulse rate.

Circulatory response The data are presented in detail in Table II and Fig. 1 and 2. The oxygen uptake (V_{O_2}) at rest in the supine position was 10.6 \pm 3.9 (range 25.0) of that predicted (Harris and Benedict 1919). During exercise V_{O_2} after 10 min work had increased to an average of 1607 ml/min (range 1300–1910) corresponding to a mechanical efficiency of 25.6 per cent (range 23.3–27.5). During the prolonged work there was an insignificant increase in V_{O_2} to an average of 1655 ml/min (range 1360–2000), corresponding to a mechanical efficiency of 24.8 per cent (range 22.9–27.1) after 50 min work.

TABLE II Data obtained at rest and during exercise with heart catheterization in 6 healthy young men

Case no	Work load kpm/min	Time min	Pulse rate, beats/min	$\dot{V}O_2$, ml STPD/min	$\dot{V}E$, l BTPS/min	O_2 cap., ml/100 ml	AVD, ml/l	Q , l/min	SV ml	Lactic acid meq/l
1	rest		68	360	10.4	19.4	48	7.4	108	0.58
	800	10	120	1,900	46.1	20.9	106	18.4	153	2.05
	800	20	122	1,930	47.0	21.1	109	17.8	146	1.60
	800	30	124	1,980	47.5	21.5	102	19.3	156	1.75
	800	40	134	2,000	50.0	21.5	102	19.6	146	1.45
	800	50	132	2,030	50.2	21.7	105	19.4	147	1.25
2	rest		61	340	9.5	18.3	43	7.8	128	0.80
	700	10	128	1,550	42.4	19.8	91	17.1	134	5.00
	700	20	130	1,580	43.3	20.0	99	16.0	123	6.45
	700	30	135	1,560	40.4	20.1	96	16.2	120	5.10
	700	40	132	1,490	40.9	20.1	102	14.6	111	4.37
	700	50	138	1,580	36.9	20.5	99	16.0	116	2.98
3	rest		77	310	8.3	19.5	40	7.6	99	1.07
	750	10	133	1,710	37.6	20.9	92	18.6	139	2.45
	750	20	140	1,750	38.8	20.6	97	18.1	129	1.75
	750	30	142	1,740	38.8	20.7	105	16.6	117	1.57
	750	40	146	1,780	39.1	20.9	102	17.5	120	1.16
	750	50	147	1,820	40.5	21.3	104	17.6	120	1.03
4	rest		62	250	6.4	18.8	40	6.2	100	0.58
	600	10	126	1,480	33.0	19.5	101	13.8	106	1.74
	600	20	130	1,470	34.4	20.3	107	12.8	99	1.42
	600	30	129	1,370	33.9	20.0	102	13.5	105	1.52
	600	40	136	1,420	39.9	20.1	106	13.4	98	1.59
	600	50	145	1,450	36.2	20.2	101	14.4	100	1.76
5	rest		72	240	7.9	16.5	43	5.5	76	0.57
	600	10	130	1,300	32.9	17.4	90	14.5	111	2.03
	600	20	133	1,310	33.9	17.4	87	15.1	114	1.54
	600	30	135	1,290	33.9	17.6	86	14.9	111	1.20
	600	40	138	1,330	33.7	17.7	87	15.3	111	1.23
	600	50	144	1,360	34.3	17.7	95	14.7	102	0.96
6	rest		70	290	9.1	17.3	37	7.9	113	1.04
	750	10	132	1,650	41.2	18.3	96	17.2	130	2.55
	750	20	136	1,650	43.2	18.3	94	17.6	130	3.00
	750	30	140	1,660	46.0	18.2	90	18.4	132	2.63
	750	40	138	1,630	43.2	17.9	89	18.4	133	2.50
	750	50	143	1,690	43.9	17.9	87	19.4	135	2.25
7	rest		70	290	9.1	17.3	37	7.9	113	1.04
	750	60	144	1,730	45.3	18.0	91	19.1	132	2.37

mm The symbols are the same as in Fig. 1—4

Pressure mm Hg

R	PA			PCV			Br A			Blood vol l	Plasma vol l
	S	D _c	S	D	M	M	S	D	M		
2	—	—	24	15	20	—	130	77	91	—	—
6	—	—	37	22	30	—	153	70	103	—	—
—	—	—	32	19	26	—	150	71	103	—	—
34	—	—	31	17	25	—	155	76	103	—	—
40	—	—	31	17	25	—	156	82	112	—	—
45	—	—	31	16	24	—	156	85	109	—	—
47	7	—	19	9	15	13	122	69	89	60	36
48	2	—	31	15	22	—	180	98	121	60	36
49	2	—	26	10	18	—	177	90	120	—	—
53	1	—	25	14	17	—	170	87	113	—	—
53	1	—	24	10	17	—	160	84	110	56	34
58	1	—	22	10	17	—	162	85	112	—	—
29	1	—	24	10	17	9	157	85	112	56	34
23	9	—	17	11	15	10	118	68	87	70	41
41	4	—	30	15	24	—	147	69	103	—	—
33	2	—	32	15	20	13	139	66	92	60	35
30	2	—	29	12	17	—	132	63	87	—	—
30	1	—	27	11	17	—	133	65	88	—	—
30	1	—	27	11	18	—	125	62	87	60	34
40	2	—	27	13	19	13	117	60	82	—	—
47	7	—	20	10	15	10	123	67	87	52	32
38	1	—	29	16	22	17	140	73	94	53	31
—	—	—	24	10	16	—	130	66	82	—	—
35	1	—	25	10	17	—	120	59	80	—	—
33	1	—	23	8	17	—	120	59	77	—	—
32	1	—	25	10	17	—	123	60	81	51	31
36	2	—	26	12	16	8	115	56	78	—	—
39	8	—	25	10	15	9	105	64	79	50	33
38	7	—	31	21	28	18	136	80	100	47	30
31	3	—	30	18	24	—	141	71	100	—	—
31	3	—	32	18	24	—	132	68	93	—	—
35	2	—	29	17	22	—	133	68	93	47	30
37	2	—	33	17	22	—	132	68	89	47	30
37	1	—	32	14	21	11	125	66	87	—	—
19	6	—	15	8	12	9	119	70	85	62	40
46	5	—	36	18	24	—	141	69	96	—	—
41	3	—	30	15	23	—	—	—	—	61	38
43	3	—	30	14	21	—	138	67	90	—	—
4	3	—	26	13	19	—	137	63	93	62	39
42	3	—	29	13	21	—	144	65	91	—	—
43	3	—	31	15	23	12	141	64	93	63	40

TABLE III. Respiratory data at rest and during exercise in 6 healthy young men. The symbols are as

Case no.	Work load kpm/min	Time min	Respiratory rate breaths/min	\dot{V}_T l BTPS/min	\dot{V} ml BTPS	\dot{V}_{O_2} ml STPD/min	RQ
1	rest		16	10.4	650	360	0.78
	800	10	22	46.1	2 100	1 950	0.83
	800	20	24	47.0	1 960	1 930	0.91
	800	30	25	47.5	1 900	1 930	0.89
	800	40	24	50.0	2 080	2 000	0.90
	800	50	26	50.2	1 930	2 030	0.88
2	rest	—	12	9.5	790	340	0.81
	700	10	18	42.4	2 350	1 350	0.99
	700	20	15	43.3	2 680	1 580	0.98
	700	30	13	40.4	3 110	1 560	0.90
	700	40	13	40.9	5 150	1 490	0.97
	700	50	10	36.9	3 690	1 580	0.86
3	rest		17	8.3	490	310	0.75
	750	10	18	37.6	2 090	1 710	0.88
	750	20	22	38.8	1 760	1 750	0.84
	750	30	18	38.8	2 160	1 740	0.87
	750	40	17	39.1	2 300	1 780	0.85
	750	50	17	40.5	2 380	1 820	0.83
4	rest		16	6.4	400	250	0.75
	600	10	15	33.0	2 200	1 480	0.91
	600	20	17	34.4	2 020	1 470	0.85
	600	30	19	33.9	1 790	1 370	0.88
	600	40	22	39.9	1 820	1 420	0.93
	600	50	19	36.2	1 900	1 450	0.82
5	rest		19	7.9	420	240	0.80
	600	10	21	32.9	1 570	1 300	0.89
	600	20	20	33.9	1 700	1 310	0.91
	600	30	2	33.9	1 540	1 290	0.88
	600	40	22	33.7	1 530	1 330	0.87
	600	50	2	34.3	1 560	1 360	0.84
6	rest		18	9.1	510	290	0.84
	750	10	23	41.2	1 790	1 650	0.94
	750	20	26	43.7	1 660	1 650	0.94
	750	30	27	46.0	1 700	1 660	0.96
	750	40	28	43.2	1 550	1 630	0.90
	750	50	28	43.9	1 570	1 690	0.92
7	rest		17	8.3	490	310	0.75
	600	10	15	33.0	2 200	1 480	0.91

* As cup-determinations with a correction of 3 mm Hg.

the same as in Fig 1--4

\dot{V}_D , ml BTPS	\dot{V}_D , l BTPS/ min	Rectal temp °C	Arterial		Stand. bicarb meq/l	\dot{V}_A , l BTPS/ min	$\frac{\dot{V}_A}{Q_C}$
			pH units	P_{CO_2} , mm Hg			
221	4.2	36.6	7.43	39*	25	6.2	0.84
274	6.9	37.0	7.42	38*	23	39.2	2.13
283	7.1	37.1	7.42	38*	23	39.9	2.24
307	8.6	37.2	7.43	39*	24	38.9	2.02
398	10.5	37.4	7.43	40*	24	39.5	2.02
423	12.0	37.5	7.43	41*	24	38.7	1.97
210	3.2	36.6	7.44	38*	24	6.3	0.81
300	7.2	37.0	7.36	38*	20	32.2	2.06
364	6.1	37.2	7.38	36*	20	37.2	2.33
364	5.3	37.4	7.38	35*	20	35.1	2.17
495	6.9	37.5	7.40	35*	21	34.0	2.33
476	5.2	37.6	7.41	37*	22	31.7	1.98
443	5.8	37.7	7.42	38*	23	30.4	1.96
160	3.4	36.9	7.39	41	23	4.9	0.64
231	4.9	37.1	7.38	40	22	32.7	1.76
313	7.8	37.3	7.39	41	24	31.0	1.71
391	7.7	37.4	7.39	42	23	31.1	1.87
443	8.2	37.5	7.40	43	23	30.9	1.77
508	9.3	37.6	7.41	42	24	31.2	1.77
338	6.4	37.7	7.42	40	23	33.8	1.96
127	2.7	36.7	7.41	44	25	3.7	0.59
312	5.2	37.1	7.42	42	24	21.8	2.01
324	6.2	37.3	7.43	39	24	28.2	2.20
388	8.1	37.5	7.43	41	25	25.8	1.91
377	9.1	37.7	7.46	37	25	30.8	2.29
303	6.5	37.8	7.46	39	25	29.7	2.06
354	8.7	37.9	7.45	39	25	27.4	2.02
151	3.6	36.5	7.39	39	21	4.3	0.58
273	6.6	36.9	7.38	39	21	26.3	1.81
313	7.5	37.1	7.40	39	22	26.4	1.75
268	6.8	37.2	7.41	37	22	27.1	1.82
327	8.1	37.4	7.40	39	22	25.6	1.67
334	8.2	37.5	7.41	39	22	26.1	1.77
304	9.6	37.6	7.40	37	21	24.8	1.72
142	3.3	36.9	7.41	37	22	5.8	0.74
282	7.4	37.1	7.34	40	20	33.8	1.96
392	9.3	37.3	7.36	42	21	31.9	1.81
420	12.4	37.6	7.36	41	20	33.6	1.82
393	12.1	37.7	7.36	41	21	31.1	1.69
321	10.1	37.8	7.37	40	21	33.8	1.74
363	10.8	37.9	7.37	40	20	34.5	1.80

The heart rate at rest was on an average 68.3 beats per minute (range 61—77). During exercise it rose to an average of 128.2 beats per min (range 120—133) after 10 min work. Continued work increased the heart rate in five-sixths of the subjects to an average of 141.5 beats per min (range 132—147) after 50 min work. In subjects who exercised for 60 min there was a further increase of, on an average, 3.4 beats per min (range 142—157).

The oxygen saturation of arterial blood was normal (mean 96.9 per cent) at rest and remained unchanged during the whole work period (97.3 and 97.2 per cent mean value after 10 and 60 min work).

The cardiac output at rest was on an average 7.1 litres per minute (range 5.5—7.9). During exercise cardiac output increased after 10 min to an average of 16.6 l/min (range 13.8—18.6). During the continued work the cardiac output showed no significant change, the mean value after 50 min being 16.9 l/min (range 14.4—19.6). All values at rest and during work fell within the normal limits predicted from the normal regression of cardiac output to oxygen uptake published from this laboratory (Bevegård *et al.* 1960).

The stroke volume at rest in supine position was on an average 104.0 ml (range 76—128). After 10 min exercise the stroke volume had increased in all subjects to an average of 128.8 ml (range 106—153), an increase of 23.8 per cent above the resting volume. In all subjects the stroke volume decreased continuously during the work period to an average of 120.0 ml (range 100—146) after 50 min, an in-

change ($0.1 > P > 0.05$). In the 5 subjects who worked for 60 min

there was a further decrease of 6 ml during the last 10 min ($P < 0.05$). The volume was, however, still larger than at rest. The 10-min values fell within the normal range of variation for the relationship between the stroke volume during exercise in supine position and total hemoglobin, heart volume and $W_{1.0}$ (Holmgren, Jonsson and Sjöstrand 1960).

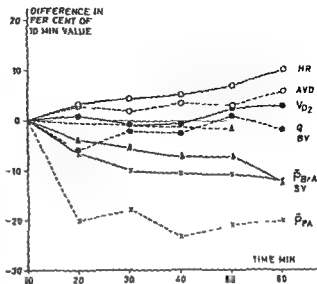
Intracardiac and intravascular pressures

The pulmonary wedge pressure (PCV) was recorded at rest in five, after 10 min in two and after 50 min in 3 subjects. At rest the PCV pressure was on an average 10.2 mm Hg (range 9—13) and after 60 min work 10.6 mm Hg (range 8—13) ($n = 5$). In two subjects the averages were 9.5 mm Hg, 17.5 and 9.0 mm Hg at rest, after 10 and 60 min work respectively.

The systolic right ventricular pressure was at rest, on an average 22.8 mm Hg (range 19—27) and rose after 10 min exercise to an average of 41.0 mm Hg (range 38—46). There was an insignificant decrease to a mean of 36.3 mm Hg (range 28—45) after 50 min work (mean change -4.7 mm Hg). The end-diastolic pressure in the right ventricle ($n = 5$) at rest was on an average 7.4 mm Hg and fell significantly ($P < 0.05$) after 10 min work to an average of 3.8 mm Hg and after 60 min work to 2.2 mm Hg, a further decrease of 1.6 mm Hg but insignificant.

The systolic pulmonary arterial pressure was at rest, on an average 20.0 mm Hg (range 15—25), rose after 10 min work to 32.8 mm Hg (range 29—37), and de-

Fig 3 Hemodynamic responses during long term exercise in supine position in six subjects. \bar{P}_{BR} = brachial arterial mean pressure, \bar{P}_{PA} = pulmonary arterial mean pressure, BV = total blood volume. The other symbols are the same as in Fig 1. Data are presented as a percentage difference from the values obtained after 10 min work.



creased after 50 min work to 27.8 mm Hg (range 22–33 mm Hg, mean change -5.0 mm Hg). The mean pressure in the pulmonary artery was, at rest, on an average 15.3 mm Hg (range 12–20), 25.0 mm Hg (range 22–30) after 10 min work and 19.8 mm Hg (range 17–25) after 50 min work, a decrease of 5.2 mm Hg between 10 and 50 min.

The decrease in mean pressure is highly significant ($P < 0.001$) and is mainly a result of a decrease in the diastolic pressure.

The systolic brachial pressure was on an average 119.5 mm Hg (range 105–130) at rest, 150.2 mm Hg (range 136–180) after 10 min work and 140.3 mm Hg (range 123–156) after 50 min work (mean change -9.8 mm Hg) with a further decrease of 6 mm Hg for the 5 subjects who worked for 60 min. The corresponding figures for diastolic pressure were 69.2 mm Hg (range 64–77), 76.5 mm Hg (range 69–98) after 10 min work and 71.3 mm Hg (range 60–88) after 50 min work. The average mean pressure was at rest 86.3 mm Hg (range 83–91), 102.8 mm Hg (range 94–121) after 10 min work and 95.8 mm Hg (range 81–115) after 50 min work (mean change -7.0 mm Hg), with a further decrease of 1.6 mm Hg for the 5 subjects who worked for 60 min. The decrease from 10–60 min is significant ($P < 0.01$). The pulse pressure increased from 52.0 mm Hg at rest to 73.7 mm Hg after 10 min work and then decreased to 71.0 mm Hg after 50 min work. The decrease in the systemic arterial mean pressure with a constant cardiac output indicates a decrease in the systemic vascular resistance.

Respiratory response

The results of the respiratory studies are presented in detail in Table III and Fig 2 and 4.

The minute ventilation (\dot{V}_E) increased from an average value of 8.6 l BTPS (range 6.4–10.4) at rest to 20.1 l BTPS after 10 min work.

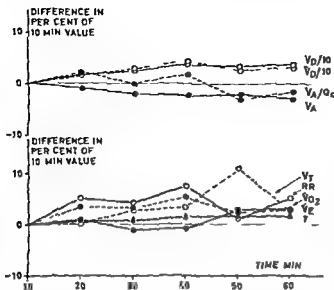


Fig. 4 Respiratory responses during long term exercise in supine position. V_D = physiological dead space ml BTPS/min. V_T = tidal volume ml BTPS/min. The other symbols are the same as in Fig. 2. Data are given in percentage difference from 10 min value.

It increased slightly to an average of 40.3 (range 34.3–50.2) after 50 min work. This increase was caused by a combined increase of tidal volume (V_T) from 2.02 l to 2.17 l between 10 and 50 min and of respiratory rate from 19.5 to 23.3 breaths/min.

Alveolar ventilation (V_A) increased from an average value of 5.2 l BTPS/min (range 3.7–6.3) at rest to an average of 32.5 l BTPS/min (range 26.3–39.2) after 10 min work. V_A remained unchanged during the continued work, mean value 31.8 l BTPS/min (range 26.1–38.2) after 50 min work, the slight increase in V_E being caused by an increase in the dead-space ventilation (V_D) due to an increase both of respiratory rate and of V_D . V_D increased from an average of 6.4 l BTPS/min (range 4.9–7.4) after 10 min to 8.6 l BTPS/min (range 5.2–10.5) after 50 min work ($P < 0.05$).

The respiratory quotient, RQ , at rest averaged 0.79 (range 0.75–0.84). After 10 min work RQ had risen to 0.92 (range 0.88–0.99). It decreased slightly to an average of 0.86 (range 0.82–0.92) after 50 min work ($P < 0.05$).

The arterial carbon dioxide tension (P_{aCO_2}) at rest averaged 39.7 mm Hg (range 37–44). During work P_{aCO_2} remained constant, on an average 39.5 and 39.3 mm Hg after 10 and 50 min work.

The pH of arterial blood was on an average 7.41 (range 7.39–7.44) at rest. After 10 min work there was a slight decrease to an average of 7.38 (range 7.34–7.42) and then a slight increase to an average of 7.42 (range 7.37–7.46) after 50 min work.

The standard bicarbonate at rest averaged 23.3 meq/l (range 21–25). It decreased slightly to an average of 21.7 meq/l (range 20–24) after 10 min work. After 50 min work the value had returned to about the same as at rest, on an average 23.0 meq/l (range 21–25).

Base excess at rest averaged -0.85 meq/l (range -4.5 – $+2.2$). During work it decreased to an average of -2.15 meq/l (range -5.0 – $+0.5$) after 10 min and to -1.45 meq/l (range -4.5 – -1.9) after 50 min.

The lactic acid concentration in arterial blood at rest averaged 1.77 meq/l. During exercise it rose to an average value of 2.60 meq/l after 10 min work and then decreased to 1.71 meq/l ($P < 0.05$) after 50 min work.

The overall ventilation-perfusion relationship of the lungs (V_A/Q_C)

The V_A/Q_C at rest averaged 0.73 (range $0.59-0.84$). After 10 min rest work the V_A/Q_C had risen to 1.96 (range $1.8-2.1$). During the continued work it showed no significant change, the value after 50 min work being on an average 1.88 (range $1.7-2.1$).

Rectal temperature

The rectal temperature at rest averaged 36.7°C (range $36.5-36.9$). During the work period there was a continuous increase to an average of 37.6°C (range $37.5-37.8$) after 50 min work, with a further increase of 0.1°C for the 5 subjects who worked for 60 min.

Discussion

The subjects used in the present investigation consisted of healthy young men (mean age 23.3 years) of ordinary physical fitness. They were slightly taller and had a higher pulse rate in standing position than the subjects used in an earlier study in the sitting position (Ekelund and Holmgren 1964). The other anthropometric data studied indicated no further differences. Because of the difficulty of exercising in the supine position the same level of working intensity as in the previous study could not be obtained without previous training of the subjects. A work load was therefore chosen which the subjects could sustain for one hour. A higher working intensity during a shorter time would perhaps show larger changes per unit time, but the accuracy of estimation of, for example, the cardiac output by the Fick method might then be reduced.

The mean pressure in the pulmonary artery decreased by 5 mm Hg (20 per cent) between 10 and 20 min work, and then remained unchanged. In the two cases when also the wedge pressures were obtained there were more marked changes in the pulmonary arterial mean pressure than in the pressure drop over the pulmonary vascular bed. A change of the arterial mean pressure in the pulmonary artery could be a result of a change of the pulmonary vascular resistance = change of capacity (expressed as change of volume per change of pressure, dV/dP) of the pulmonary vascular bed, an altered distribution of the blood volume between the systemic and lesser circulation, or a change in the intrathoracic mean pressure as a result of an altered thoracic mid position. From the observation of the wedge pressures a change of resistance does not seem to be the major factor. The absolute intrathoracic pressure level is not known in the present study. But if it is unchanged two main factors remain, a changed capacity of the pulmonary vessels and changed distribution of the blood volume.

The pressure in the brachial artery showed a different pattern of resp

a continuous decrease which was a result of a corresponding decrease of the resistance in the systemic circulation

The size of the stroke volume is dependent on the dimensions of the heart and on the emptying and filling of the ventricles. The filling of the ventricles is dependent on the available energy (kinetic and potential) and on the time for filling, and also on the mechanical properties of the myocardium. The increase of the stroke volume from rest to 10 min work (+ 23.8 per cent) is due to a change of filling energy already at rest as a result of elevation of the legs and, at least in part, therefore, should be regarded as a change of 'base line' (Frick and Somer 1964). At the start of exercise there is also the positive effect of the muscular pump on the venous vessels in the legs. The decrease in stroke volume during the continued work is the result of a combination of several of the above-mentioned factors. With the compensatory increase in heart rate the result will be an unchanged cardiac output, which is fundamental for the maintenance of the optimal relation between cardiac output and oxygen consumption during work.

A decrease in blood volume on the filling side of the heart could not be a result of a decrease in the total blood volume but must be due to an altered distribution of blood within the capacity vessels, as suggested by the change in the regional perfusion indicated by the increase in dead space ventilation.

With the decrease of arterial mean pressure there will also be some chronotropic influence from the baroreceptor system with an acceleration of the heart rate, giving a shorter filling period. Influences from the baroreceptor system or the central nervous system may also have a negative inotropic effect on the heart muscle, with a change in the myocardial function. Therefore for a certain level of work, there will be an optimal combination of filling energy and filling time. The change in one of these components will change the steady state to another level of optimal combination.

At the end of long continuous work there may be a change in cardiac metabolism with a greater part of the energy taken from an increased uptake of glucose, breakdown of glycogen and an increased production of lactate. Such a metabolic change may result, among other things, from an increased sympathetic influence on the heart.

The minute ventilation increased slightly during the continued work but the alveolar ventilation was unchanged, the increase being therefore due to increased dead space ventilation. The overall ventilation-perfusion relationship of the lungs was unchanged during the continued work, but with the increase in dead space there might be a small change in the regional ventilation-perfusion relationship. The altered total distribution of the blood which is of significance for filling of the heart, might affect the regional distribution to different parts of the lung less in the supine than in the sitting position, which would result in much smaller changes in dead space ventilation. The baroreceptor system especially in the pulmonary vessels, could be responsible for some of the changes in ventilation during the prolonged exercise, i.e. increase of respiratory rate (Kinnison *et al.* 1965).

Comparison with the study in sitting position (Ekelund and Holmgren 1964). The results differed in the experimental conditions, with a lower absolute and relative work level in the supine position. The relative intensity of exercise can be expressed by the pulse rate after 10 min and indicates a load in relation to the normal capacity of the individual in a short exercise test. The relative level of work was lower in the supine than in sitting position with a 10 minute heart rate of 128 beats/min and 148 beats/min, respectively. The absolute load was 700 kpm/min in the supine and 920 kpm/min in sitting position. The difference in circulatory reaction could therefore be due both to different postures and/or different relative working intensities. In spite of the better possibilities of filling of the heart in the supine position with the legs elevated, there was a decrease in stroke volume which, however, after the same work period (50 min), was less in supine than in sitting position. There was also a fall in the systemic pressure and a pronounced fall in the pressure in the pulmonary circulation. The decrease of stroke volume, in spite of the better possibility of filling of the left ventricle, seems to be a result of a change in tonus of the peripheral vessels with an altered distribution of blood. There may also be a change in the contractility with impaired systolic emptying of the heart as a result of a decreased sympathetic influence or of a less powerful response to the same sympathetic influence. In short time exercise Bevegård, Holmgren and Jonsson (1962) have shown that there are great differences in the circulatory adaptation during work in sitting and supine position. To evaluate how large the influence of posture is on the adaptation during prolonged exercise one has to perform a study in sitting position at about the same relative working intensity as in the supine exercise.

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Nervous Regulation of Blood Flow in the Subcutaneous Adipose Tissue in Dogs

By

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Abstract

NGAI, S. H., S. ROSELL and L. R. WALLENBERG. Nervous regulation of blood flow in the subcutaneous adipose tissue in dogs. Acta physiol. scand. 1966 68 397-403.

The effect of sympathetic stimulation on the blood flow in the subcutaneous adipose tissue was studied in dogs. The main blood flow in the adipose tissue was measured by the ^{133}Xe method. The peripheral blood flow in the skin was measured by the ^{133}Xe method. The blood flow in the adipose tissue was found to be related to the peripheral blood flow in the skin. The blood flow in the adipose tissue was found to be related to the peripheral blood flow in the skin.

was also related to the stimulus frequency. Propranolol, a β receptor blocking agent, abolished the vasodilator response. Reflex adjustment of the peripheral vasomotor tonus upon carotid artery occlusion and activation of the sympathetic cholinergic vasodilator nerves in the hypothalamus did not appreciably change the vascular resistance in the adipose tissue.

Quantitative measurements of blood flow in the adipose tissue are lacking probably due to the fact that this tissue is diffusely located between other tissues. The juxtaposition of adipose tissue and skin may make it difficult to determine its metabolism and blood circulation *in vivo*. Recently we have succeeded in isolating canine subcutaneous adipose tissue with an intact blood and nerve supply (Oro, Rosell and Wallenberg 1965). This paper will report investigations on the nervous control of blood flow in canine adipose tissue. Studies on the release of free fatty acids (FFA) from the adipose tissue with the same type of preparation are being reported elsewhere (Rosell 1966).

Methods

Female dogs weighing 8 and 14 kg were used. Pentobarbitone sodium was used as anaesthetic. The peripheral blood flow in the skin was measured by the ^{133}Xe method. The blood flow in the adipose tissue was measured by the ^{133}Xe method. The blood flow in the adipose tissue was found to be related to the peripheral blood flow in the skin.

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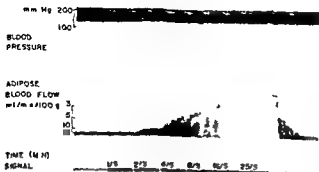


Fig. 1. Blood flow in subcutaneous adipose tissue in dog. Effects of electrical stimulation of the nerve to the adipose tissue. Stimulus parameters: 8 V, 5 msec, frequency as indicated.

medicated with morphine sulfate (10–15 mg/kg s.c.) and subsequently anesthetized with α -chloralose (100 mg/kg i.v.). The trachea was cannulated and artificial respiration provided with a Palmer Ideal pump when indicated. Heparin was given to prevent clotting.

The in vivo venous adipose tissue preparation is published elsewhere (Ngai, 1970).

and directing the blood through a silicone-oil filled cannula. In 5 expts muscle blood flow was also measured. In these experiments the artery supplying the muscle was placed in the femoral artery to the adipose tissue. The

stimuli consisting of square wave pulses (15–8 V) and varying frequency were delivered from a Grass S 4 stimulator via bipolar electrodes (0.5 mm) inserted into the brain through small burr holes. The Horsley–Clarke technique (The

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Results

Resting blood flow and effects of nerve stimulation

In each experiment the resting flow in denervated adipose tissue was determined prior to any stimulation. The mean value of blood flow from 17 expts was 8.5 ml/min \times 100 g adipose tissue and varied between 3 and 19 ml/min \times 100 g provided the arterial blood pressure was kept within its normal range. Stimulation of the appropriate sympathetic chain, or the nerve supplying the adipose tissue, decreased the blood flow, i.e. vasoconstriction occurred, the magnitude of which was frequency dependent. Fig. 1 shows the vasoconstrictor responses to electrical stimulation of the mixed nerve to the adipose tissue. Stimulation with 1/sec did not induce any significant change in the blood flow while an appreciable vasoconstriction occurred

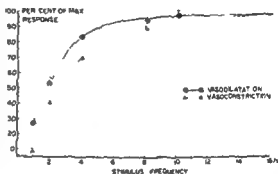


Fig 2

ergotamine was given i.a. prior to the recording. Stimulus parameters: 8 V, 3 msec frequency as indicated.

BLOOD FLOW IN SUBCUTANEOUS ADIPOSE TISSUE IN DOG AFTER 100 µg DIHYDROERGOTAMINE i.a.

Fig 3 Stimulus response curves. Electrical stimulation of the nerve to the subcutaneous adipose tissue in dog. The vasomotor reactions are expressed in percent of the maximal response obtained in each individual experiment. \blacktriangle Vasoconstrictor responses (mean of 9 expts). The sequence of stimuli was 1, 2, 4, 8 and 16/sec for 1 min at each frequency. \bullet Vasodilatation responses (mean of 5 expts). 100 µg dihydroergotamine was administered i.a. prior to the stimulus series. The sequence of the stimuli was the same as for the vasoconstrictor responses. The vertical bars represent \pm the standard error of the mean.



higher frequencies with a maximal effect being obtained with a stimulus frequency of about 16/sec. Upon cessation of the stimulation the blood flow promptly returned to control values or, in most experiments, increased to values larger than control. This secondary vasodilatation, if it occurred, was long lasting. The vasoconstrictor responses to nerve stimulation are summarized in Fig 3. It is seen that 1/sec did not produce any appreciable change in blood flow while stimulation with 2/sec induced approximately 40 per cent of the maximal vasoconstriction obtainable. The vasoconstricting effect of nerve stimulation was abolished by the i.a. injection of a receptor blocking compounds such as dihydroergotamine (20 100 µg) and phentolamine (100 µg).

Following β receptor blockade nerve stimulation invariably increased the blood flow. Fig 2 illustrates one of these experiments in which dihydroergotamine was administered i.a. prior to the recording. Stimulation with 1/sec significantly increased the blood flow above the control value with nearly a 4 fold increase occurring with stimuli of 4–16/sec. The relationship between the vasodilatation and the stimulus frequency is illustrated in Fig 3. Although nerve stimulation also



Fig 4 Blood flow in subcutaneous adipose tissue in dog. 100 μ g dihydroergotamine was given i.a. prior to the recording. At 1 and 2, electrical stimulation of the nerve to the tissue with 3/sec and 5/sec respectively. At 3 i.a. injection of 0.1 μ g acetylcholine.

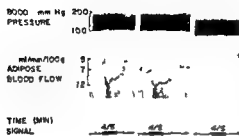


Fig 5 Blood flow in subcutaneous adipose tissue in dog. Electrical stimulation of the nerve to the adipose tissue. Dihydroergotamine 100 μ g was administered prior to the recording. Between the first and the second stimulations 100 μ g atropine injected i.a. Between the second and the third stimulations 125 μ g propranolol injected i.a. Stimulus parameters 5 \times 10 msec 4/sec.

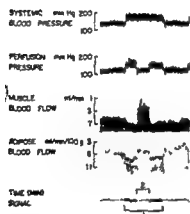


Fig 6 Blood flow in skeletal muscle and subcutaneous adipose tissue in dog. Bilateral vagotomy. 1 Bilateral carotid occlusion. 2 Perfusion pressure adjusted to the pre-occlusion level during carotid occlusion.

produced a vasodilatation after α -receptor blockade, the magnitude of change varied considerably from animal to animal. In order to relate the effect of nerve stimulation to the maximal blood flow capacity of the vascular bed in the adipose tissue large doses of acetylcholine were injected i.a. to relax all smooth muscles in the vascular bed. The increase in blood flow produced by acetylcholine was then compared with the maximal response to nerve stimulation. Fig 4 shows that nerve stimulation increased the blood flow to approximately the same degree as the i.a. injection of acetylcholine with maximal blood flow value of about 25 ml/min \cdot 100 g adipose tissue.

The vasodilator response to nerve stimulation following α -receptor blockade was abolished by the administration of propranolol, a β receptor blocking drug. Atropine had no effect in this respect (Fig 5).

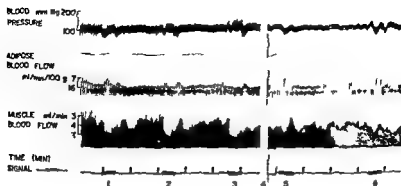


Fig 7 Blood flow in skeletal muscle and subcutaneous adipose tissue in dog. Electrical stimulation of the sympathetic cholinergic vasodilator outflow in the hypothalamus. Stimulus parameters: 3 msec, 80 sec.

1	stimulation 1	4	0.5 mg atropine i.v.
2	" 15	5	stimulation 15
3	" 20	6	" 20

Effects of reflex activation of vasomotor nerves

Occlusion of the common carotids was performed in 4 vagotomized animals in order to study the effect of reflex activation of the sympathetic outflow to the adipose tissue. Blood flow in skeletal muscle was also measured in these experiments for comparative purposes. Fig 6 illustrates one experiment in which carotid occlusion increased the mean arterial pressure from 125 to 170 mm Hg. Blood flow increased in both the adipose tissue and the muscle. However, vasoconstriction in the muscles became evident when the perfusion pressure was adjusted to the pre-occlusion level by tightening the screw clamp around the aorta. The adipose tissue blood flow was not significantly different from the pre-occlusion value and a vasoconstriction of minor degree was observed in only one experiment. In 4 animals the mean reduction in the blood flow in the muscles with constant perfusion pressure was about 50 per cent during carotid occlusion.

Effect of activation of the cholinergic vasodilator nerves

The cholinergic vasodilator outflow in the anterior hypothalamus was stimulated electrically in 3 expts in order to further study the vasomotor regulation of the vascular bed in adipose tissue. One of these experiments is illustrated in Fig 7. Stimulation resulted in an increased blood flow in the skeletal muscle. Adipose tissue blood flow remained practically unaffected during

stimulation. However, a slight blood flow decrease may be noticed which is possibly due to changes in the arterial blood pressure. The cholinergic nature of the vasodilator response in the muscle was indicated by the blocking action of atropine.

Discussion

The present study indicates that the subcutaneous adipose tissue in dogs has a resting blood flow which is of the same order of magnitude as found under similar experimental conditions in the skeletal muscle. Histological studies have shown a rich capillary network around fat cells. Gersh and Stull (1945) found in freeze dried rat adipose tissue that all fat cells were in contact with at least one capillary. Later, Held and Rutishauser (1960) produced circulatory stasis to reveal vessels in the adipose tissue and observed an abundant vascular network. Thus, the relatively high resting flow in the adipose tissue, as found in the present experiments is consonant with histological findings. Larsen, Lassen and Quaade (1966), using local clearance of Xe^{133} found an average blood flow of 2.6 ml/min/100 g in the subcutaneous adipose tissue in man which is comparable to the blood flow in resting skeletal muscles in human subjects (see Shepherd 1963). Thus, in both dogs and man the resting blood flow seems to be of the same order of magnitude in the skeletal muscle as in the subcutaneous adipose tissue in the respective species. In the present experiments stimulation of the sympathetic outflow or the nerve supplying the adipose tissue produced vasoconstrictor effects which were abolished by sympathetic agents with α receptor blocking properties. These findings indicate that vasoconstrictor nerves are sympathetic in origin and adrenergic in nature. They also show that the relationship between the stimulus frequency and the vasoconstrictor response is similar to that found in other sympathetically innervated tissues such as the nictitating membrane (Rosenbluth 1950) and the blood vessels of the skeletal muscle and the skin (Folkow 1952, Celander and Folkow 1953).

Nerve stimulation also elicited a vasodilator response provided that the vasoconstriction was blocked by α receptor blocking substances. Even though sympathetic cholinergic vasodilator fibers are known to supply vascular beds in the skeletal muscle, and possibly the heart (Ulnas 1960) the adipose tissue is apparently not innervated by this type of fiber since activation of the sympathetic cholinergic vasodilator outflow did not produce any vasodilator response (Fig. 7). Moreover, the vasodilator response induced by nerve stimulation was not blocked by atropine, but was abolished by propranolol a β receptor blocking agent (Fig. 5). This finding indicates that although the vasodilator response is adrenergic it may be considered as a β receptor effect. In this respect the vasodilator response is similar to the release of free fatty acids from the subcutaneous adipose tissue following sympathetic nerve stimulation (Fredholm and Rosell 1966b). Whether vasoconstriction and vasodilatation are due to stimulation of the same or separate adrenergic nerves remains unanswered and requires further investigation (see Discussion, Rosell 1966).

A comparison of the increased blood flow resulting from either nerve stimulation or a injection of acetylcholine reveals that nerve activity can produce a maximal

vasodilatation in the adipose tissue. This indicates that the vasodilatation occurs in the resistance section of the vascular bed, i.e. arterioles. A detailed study of the effects of nerve stimulation on consecutive sections of the vascular bed of subcutaneous adipose tissue will be published elsewhere (Öberg and Rosell 1966).

The vascular bed in the subcutaneous adipose tissue does not seem to participate to a significant extent in the reflex adjustments of the peripheral resistance elicited via the carotid baroreceptors. This is evident from the finding that occlusion of the carotid artery did not appreciably increase the vascular resistance in adipose tissue. In this respect the vasomotor reaction in the subcutaneous adipose tissue seems to be similar to that found in the kidney (Lofving 1961).

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Analysis of Odour Similarities from Electrophysiological Data

By

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Abstract

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probability values were calculated for all pairs in a given series. The statistical values were regarded to represent measurements of relatedness in olfactory stimulative properties between the tested odours. For each series the data were analysed with a nonmetric multidimensional scaling method to give spatial representations of the relatedness between the odours. The relation of the results to psychophysical findings and the classification of odours according to the stereochemical theory are discussed.

Increased knowledge about the organization of the atoms in odorous molecules has focused attention on stereochemical properties as important parameters in determining the quality of the odours. The similarity in odour quality between substances of similar molecular size and shape has frequently been reported (Moncrieff 1951, Stoll 1957, Ruzicka 1957, Beets 1957, for review see Beets 1964). According to a specific stereochemical odour theory recently proposed by Amoore (1962) there are seven primary groups of odours. Five of these groups (camphoraceous, ethereal, floral, minty and musky) are based upon the steric resemblance of the substances within each of the groups. The classification is made from common semantic descriptions of odours. Although these descriptions as such may give valuable qualitative information they do not provide quantitative measurement of relatedness between the odours. It is possible, however, by various available methods to obtain quantitative information about the relatedness between different odours (see Discussion). The psychological studies used to test the stereochemical theory of olfaction have provided evidence for a significant correlation between 'similarity of odour' and

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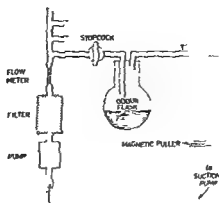


Fig 1 Schematic drawing of the stimulating apparatus (see text)

'similarity of shape' (Amoore and Venstrom 1966, Amoore 1966). The present study is an extension of previous electrophysiological investigations (Doi and Amoore 1966) and was undertaken with the intention to obtain data on the relationship between the stimulative properties of some odorous substances selected from various groups of Amoore (1962). A statistical method of processing the electrophysiological data is used and the results are compared with those obtained by psychophysical methods. In addition the statistical data are analyzed to give spatial representation of the relatedness of the different odours.

Methods

The skull was covered with a layer of paraffin and the side was opened.






The epithelium were removed and the agar in Ringer's solution was removed.



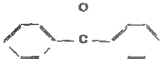

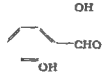


The bulb was then filled with the odorant solution.





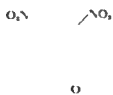

The bulb was then placed in the tube and the tube was held in place by the experimenter. The duration of the stimulation was controlled by the experimenter.

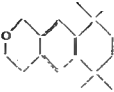
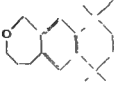


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TABLE I Substances used in the present experiments. The impurities are given in per cent of the total amount of substance present

Substance	Structural formula	Source	Impurities (per cent)
Camphoraceous			
1 p-Dichlorobenzene		Hopkin & Williams England	0.6
2 d, 1-Camphor		Hopkin & Williams England	0.2
3 1, 8-Cineole		E. Merck, Germany	0.2
4 2-Methyl 2-butanol	$\begin{array}{cccc} \text{CH}_3 & \text{CH}_2 & \text{COH} & \text{CH}_3 \\ & & & \\ & & \text{CH}_3 & \end{array}$	Hopkin & Williams England	< 0.1
5 3-Methyl 2-butanone	$\begin{array}{cccc} \text{CH}_3 & \text{CH} & \text{CO} & \text{CH}_3 \\ & & & \\ & \text{CH}_3 & & \end{array}$	Mallinckrodt USA	1.1
6 2-Methyl 2-propanol	$\begin{array}{ccc} \text{CH}_3 & \text{COH} & \text{CH}_3 \\ & & \\ & \text{CH}_3 & \end{array}$	Hopkin & Williams England	0.1
Ethereal			
7 1, 2-Dichloroethane	$\text{ClH}_2\text{C}-\text{CH}_2\text{Cl}$	Hopkin & Williams England	0.2
8 Carbon tetrachloride	C Cl_4	E. Merck Germany	0.1
9 Furan		Hopkin & Williams England	—
10 1-Propanol	$\text{CH}_3-\text{CH}_2-\text{CH}_2\text{OH}$	KERO, Sweden	2.3
11 Pyrrole			< 0.1

Substance	Structural formula	Sn	
12 Trichloroethene	$\text{CHCl}=\text{CCl}_2$	E N	(
Floral			
13 Acetophenone		Hopkin & Williams England	0.8
14 Anisole		Hopkin & Williams England	
15 Benzophenone		Hopkin & Williams England	0.1
16 Geraniol		British Drug House	10.0
17 Salicylaldehyde		Hopkin & Williams England	0.1
Minty		Hopkin & Williams England	0.1
18 Cyclopentanone			
19 Cycloheptanone		Fluka, Switzerland	2.5
20 Cyclooctanone		Koch Light Lab England	2.1

Substance	Structural formula	Source	Impurities (per cent)
21 Diethylsulphate	$(\text{CH}_3\text{CH}_2)_2\text{SO}_4$	Hopkin & Williams England	1.3
22 1-Menthol		Route Bertrand Dupont, France	pure
23 1-Menthone		Route Bertrand Dupont, France	pure
24 neo-Menthol*		Route Bertrand Dupont, France	pure
25 neo-iso-Menthol*		Route-Bertrand Dupont, France	pure
Musky			
5 α -Androstan-3 β -ol		Koch Light Lab England	10.0
27 Celestolide* (1,1-dimethyl-6- <i>tert</i> - butyl-4-acyl indan)		International Flavors & Fragrances The Netherlands	pure
28 Musk ketone (2,6-dimethyl-4- <i>tert</i> - butyl-3,5-dinitro- acetophenone)		International Flavors & Fragrances The Netherlands	pure
29 Musk lactone (15- hydroxypenta- decanone acid lactone)		International Flavors & Fragrances The Netherlands	pure

Substance	Structural formula	Source	Impurities (per cent)
10 Musk I* (2-oxa 5,5,8,8-tetramethyl-1,2,3,4,5,6,7,8-octahydroanthracene)		International Flavors & Fragrances The Netherlands	pure
11 Musk B* (6,7-[4-oxacyclohepteno]-1,1,4,4-tetramethyltetralin)		International Flavors & Fragrances The Netherlands	pure
12 Musk A* (2-oxa-1,5,5,8,8 penta methyl 1,1,2,3,4,5,6,7,8-octahydroanthracene)		International Flavors & Fragrances The Netherlands	pure
13 Musk T* (1,1,6-trimethyl 7-isopropyl 5 acetyl tetralin)		International Flavors & Fragrances The Netherlands	pure

marked with an asterisk these substances were chosen because of their resemblance to other chemicals within the particular groups and because they could be obtained in a very pure state. The substances which were not especially purified for these experiments were tested for impurities by gaschromatography. As seen from Table I most substances had impurities which were less than one per cent. Pyrrole was obtained newly prepared and had only a small amount of impurities when tested a year after preparation and usage. The sample of furane had evaporated before it could be tested. 2 Methyl 2 butanol contained about 7 per cent impurities. The sample of 5 α androstan 3 β -ol contained 10 per cent of another substance, most probably androstanone.

TABLE II Chi square matrix for the 'camphoraceous' substances

	2	4	5	6
1 p-Dichlorobenzene	0.101	5.973	11.035	3.237
2 d, l-Camphor		0.542	10.036	5.702
4 2-Methyl-2-butanol			8.471	15.864
5 3-Methyl-2-butanone				13.569
■ 2-Methyl-2-propanol				

tions (Kruskal 1964 a, b). In many cases the three-dimensional solution was superfluous, in one case it had a higher 'stress' than the two-dimensional one. The only exceptions were the two arrays of data from the studies with odours of the 'cross comparison' and the 'musky' substances. One of the one-dimensional solutions had acceptable 'stress'.

This method of analyzing the spatial arrangement of the comparisons between odours is based upon the rank order between the pairs rather than upon actual measurements. The distances in one figure are therefore not comparable to the distances in any other figure.

Results

Units in the frog's olfactory bulb show irregular spontaneous activity. Stimulation of the receptor epithelium with odours most frequently caused an inhibition of their activity, and only about 20 per cent of the units were excited by stimulation. The relative number of excited units was lower in the present study than in the previous ones (Doving 1964, 1965, 1966), mostly on account of the larger number of units inhibited. The relative number of units unaffected was about the same as in the previous studies. A total number of 500 units were studied.

'Camphoraceous'. A total of 83 units were studied for the camphoraceous odours. Of these units 16 per cent were excited, 73 per cent inhibited, and the rest unaffected by stimulation.

The chi-square matrix is shown in Table II. The highest chi-square value was obtained for the pair of 2-methyl-2-propanol and 3-methyl-2-butanol. The lowest values were found for the 3 pairs p-dichlorobenzene and d,l-camphor, d,l-camphor and 2-methyl-2-butanol, and p-dichlorobenzene and 2-methyl-2-propanol. These pairs were the only ones which gave chi-square values indicating independence.

The two-dimensional solution for the camphoraceous substances based upon the data in Table II is given in Fig. 2. This configuration of the 5 odours has zero stress,

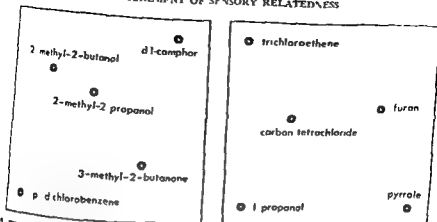


Fig 2 The two dimensional configurations of the odours used in the camphoraceous (left) and ethereal (right) Zero stress for both solutions

Table III Chi-square matrix for the ethereal substances

	9	10	11	12
9 Carbon tetrachloride	17 554	10 668	8 272	18 615
10 Furan		4 762	9 819	5 151
11 Propanol			1 211	3 437
12 Pyrrole				1 059
12 Trichloroethene				

compared with 19 per cent stress for the one dimensional solution. As seen from the figure the alcohols and ketone were placed in the center of the configuration with p-dichlorobenzene and camphor at each side. The monotonicity of the function relating the data to interpoint distances was achieved by equalizing five of the values. Whether this is inherent in the data or is a result of the method of analysis is not clear.

Ethereal The substances used for representing Moore's ethereal group excited in average 20 per cent and inhibited about 70 per cent of the 100 units studied. Trichloroethene excited about 25 per cent of the bulbar neurones. All the substances except propanol evoked unusual electro olfactograms with initial positive deflections (cf. Bykov and Fierova 1964; Gesteland *et al.* 1965) most probably due to their deleterious and narcotic effects upon the olfactory cilia. These effects made it difficult to estimate the strength of each stimulation and hence to judge the responses of the bulbar units at the same stimulating efficiency.

The chi square values were generally higher than in the camphoraceous group (see Table III). Of the 10 pairs seven gave chi-square values indicating that the odours were significantly associated at a 5 per cent level. Only the pairs between propanol, pyrrole and trichloroethene gave chi square values indicating independence.

TABLE IV Chi square matrix for the floral substances

	14	15	16	17
13 Acetophenone	6.291	0.005	0.231	10.418
14 Anisol		0.441	4.671	0.234
15 Benzophenone			0.176	0.187
16 Geraniol				0.342
17 Salicylaldehyde				

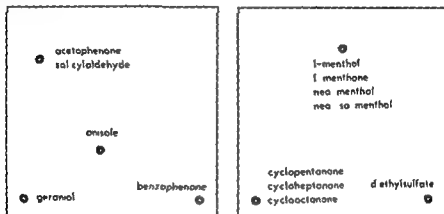


Fig. 3 The two-dimensional configurations of the odours used in the floral (left) and minty (right) series. Zero stress for both solutions.

The lowest value was found for the pair pyrrole-trichloroethene. The structure in the data of Table III is illustrated in Fig. 2. This configuration has zero stress.

Floral. The substances selected from Amoore's floral group excited an average of 15 per cent of the bulbar units and inhibited about 77 per cent.

The chi square values were much lower than in the series mentioned above (see Table IV). Significantly high chi square values were found only for the pairs acetophenone and anisole, acetophenone and salicylaldehyde, and anisole and geraniol.

The two-dimensional solution of the data with floral substances is given in Fig. 3. The points representing acetophenone and salicylaldehyde fell together. This two-dimensional solution also had zero stress.

'Minty.' A relatively larger proportion (83 per cent) of the 91 bulbar units was inhibited by the substances representing Amoore's peppermint group than by the substances from other series.

As seen from Table V, the l-menthol and menthol derivatives made pairs with high chi square values, seemingly constituting a homogenous group of odours. A similar group was made up by the cycloketones, between which high chi square values were also found. Most of the other pairs did not show a significant association.

TABLE V Chi-square matrix for the 'minty' substances

	19	20	21	22	23	24	25
18 Cyclopentanone	17.605	7.010	4.175	0.079	4.188	1.535	6.549
19 Cycloheptanone		8.496	0.008	1.784	6.074	6.567	6.865
20 Cyclooctanone			4.945	0.232	2.398	0.184	2.562
21 Diethylsulphate				0.059	0.009	0.164	0.378
22 l-Menthol					18.836	24.759	13.575
23 l-Menthone						7.261	54.712
24 neo-Menthol							8.891
25 neo-iso-Menthol							

TABLE VI Chi square matrix for the 'musky' substances

	27	28	30	31	32	33
26 5 α -Androstan 3 β -ol	12.229	3.490	3.593	14.399	4.721	11.423
27 Celestolide		14.896	8.333	25.357	9.797	8.494
28 Musk ketone			4.989	5.980	7.746	13.516
30 Musk I				4.723	15.552	10.556
31 Musk B					7.506	7.386
32 Musk A						6.054
33 Musk T						

The configuration of the eight minty odours in two dimensions is illustrated in Fig. 3. This solution had zero stress while the three- and one-dimensional solutions gave stress factors of 0.5 and 27.3 per cent respectively. The coincidences of the menthol derivatives of the cycloketones shows the similarities within these odour groups. The three interpoint distances are equal and the distances of diethylsulphate from the other odours are the same as the distance between the menthol and cycloketones. As in the camphoraceous case the monotonicity of the function relating the chi-square values to interpoint distances was achieved by equalizing the distances between the cycloketones and between the menthol derivatives.

Musky. The proportion of units excited by the musky substances used in this study was about the same as in the previous experimental series. The relative number of units inhibited by these odours was however smaller than in other series on account of a larger number of units being unaffected by stimulation. The receptor potentials evoked by stimulation with these substances had slower time courses and were of relatively smaller amplitudes than those elicited by substances used in the other experiments. There might be a relationship between the large number of unaffected units and the relatively small amplitude of the EOG elicited by these musky odours.

The chi-square values given in Table VI indicate a significant association between all odours in this series. Androstan 3-ol most frequently gave pairs with 1 square values, thus indicating a slight dissimilarity from the other odour

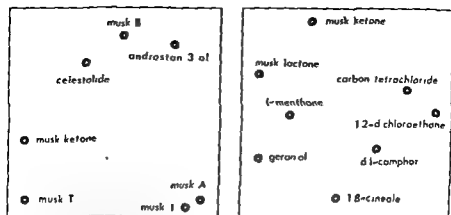


Fig 4 The two-dimensional configurations of the odours used in the 'musky' series (left) and 'cross comparison' (right) Stress 10.9 and 14.6 per cent respectively.

TABLE II

	3	7	8	16	23	28	29
2 d, 1 Camphor	1.514 0.9	4.165 6.5	1.222 5.7	0.657 5.2	3.618 6.2	0.021 5.8	0.790 6.7
1.8 Cineole		1.156 7.3	0.035 6.0	2.233 4.8	0.022 6.5	0.090 5.6	0.048 7.6
7 1, 2 Dichloroethane			8.342 1.7	0.000 6.7	0.097 8.0	0.046 7.0	0.005 8.1
8 Carbon tetrachloride				0.970 5.6	0.145 6.6	0.026 5.9	0.312 6.9
16 Geraniol					2.398 5.5	0.870 2.8	2.247 4.3
23 1 Menthone						0.194 6.6	2.097 7.7
28 Musk ketone							5.847 1.9
29 Musk lactone							

The configuration of the seven 'musky' odours given in Fig 4 has 10.9 per cent stress which is 'fair' according to the terminology used by Kruskal (1964a). The three-dimensional solutions had zero stress while the solution in one dimension had 29 per cent stress.

'Cross-comparison' The substances used in this series were selected in collaboration with Amoore from his 5 groups to make possible a comparison between the results of his psychophysical studies (Amoore and Vinstrom 1966, Amoore 1966, and personal communication 1965) and the results from these electrophysiological experiments.

A total of 97 units were tested for these eight odours. An average of these were excited and 75 per cent inhibited.

The chi square values for pairs of these substances were not significantly different from the previous series (Table VII). The pairs 1,2-dichloroethane, and 1,2-dichloroethane and camphor gave the same values. Most chi square values indicated independence between these two series.

The probability values showed greater divergence from the null hypothesis than in any other experimental series judged by the rank correlation coefficient. The rank correlation coefficient was, however, significant at the 0.05 level. The underlying structure of the data for the odours of the series is given by the configuration of Fig. 4. This solution shows a clear stress which is between 'fair' and 'poor' according to the term of Kruskal (1964 a). The three-dimensional solutions had 6.3 per cent of the variance explained. A smoother function relating the chi-square values to interpoint distance was found. The solutions showed any tendency to group the odours.

Discussion

Various psychophysical methods have been used to obtain measurements of the sensory relatedness between odours. The easiest and simplest method to perform is seemingly the one based upon quantitative comparison of similarity estimates (Ekman 1954, Engen 1962). Modifications of this principle have been developed by other authors, whose studies have given a large amount of data (Schutz 1964, Wright and Michels 1964, Yoshida 1964, Lange 1966, Amoore and Ventrom 1966). Another method, the so-called 'cross adaptation', is based upon the fact that adaptation to one odour causes fatigue to other odours. Since the effect is selective the degree of similarity can be estimated by measuring the extent of threshold variations (Zwaardemaker 1907, Backman 1917, Le Magnen 1948, Cheesman *et al.* 1953, 1956, Engen 1963, Koster 1965). The confusion principle was first used in olfactory investigations by Le Magnen in 1950, in studies on odour discrimination in rats. The same method has later been applied by Lange (1966) in studies on human subjects. Measurements of sensory relatedness based upon electrophysiological data have been obtained by the present author (Doving 1965, 1966). The method is based upon categorizing the effects on the units in the olfactory bulb in this way the data may be subject to enumeration statistics. The chi square values and the distribution probability can be calculated for any two pairs of odours in an experimental series. The results from the electrophysiological studies are in accordance with psychophysical studies for the odour substances selected from a homologous series (Engen 1962, Doving 1966). The consistency between the results of various psychophysical and electrophysiological studies is statistically significant even for non homologous substances which are very different in odour quality (Amoore 1965, Doving and Lange 1966). The substances used in the 'cross-adaptation' series provided data for a comparison between the present chi square

the method used by Amoore and Venstrom (1966). The results show a significant rank correlation coefficient (0.498) when compared with Amoore's data (Table VII, personal communication 1965). The probability of obtaining such a good correlation by chance alone is less than one per cent. The implications of these agreements are further discussed by Doving and Lange (1966).

The 'musky' odours seem to be a rather homologous group with significantly high chi square values between all pairs. The rather strong relationship between the musky odours indicated by the present findings is interesting in relation to the studies of Guillot and Le Magnen. Le Magnen (1948) showed that adaptation to a macrocyclic musk (musk lactone) did not influence the threshold to a keto musk. Consistent findings on humans with partial anosmia were reported by Guillot (1948 a, b) who found subjects with very high thresholds for a few musks but normal thresholds for all other musk tested. These authors have concluded that the olfactory epithelium contain different types of receptors sensitive to these individual musks, even though they constitute a homogenous group when judged by subjective standards. As demonstrated by the present findings the chi square values are consistent with the results of subjective judgements.

According to the stereochemical theory of olfaction (Amoore 1962) the spatial arrangement of the atoms in the odorous molecules are the most important factors determining odour quality. Amoore has classified a large number of odours in seven groups, 5 of which are determined by the stereochemical properties of the substances. For two groups (putrid and pungent) Amoore suggests that the overall charge of the molecule is the important parameter for quality determination. The concrete form of this stereochemical theory makes it especially valuable and subjects it to test (cf. Amoore and Venstrom 1966; Amoore 1966). In the present study the sensory relatedness of the odours in a series have been estimated. The odours were selected from five of Amoore's groups and if the classification is valid the results should be consistent with the theory.

A reasonable assumption would be that for all pairs of odours within a certain group the measurements of association should be higher than between odours from different groups. Similar assumptions should hold when the odours are plotted in a spatial representation of their relatedness. Within the spatial configuration the distances between odours of one particular group should always be smaller than the distances between odours of different groups. As seen from the present results these assumptions were fulfilled in some cases but not in others. For example between substances of the floral group. Neither were substances from different groups always independent as seen from the cross comparison. In the camphoraceous, ethereal and musky series the chi square values found were comparatively large but some odours gave small chi square values indicating independence. In the minty series the cycloketones and menthol derivatives gave high values while small values were found between odours of these two groups. The spatial representation also reflects these results by placing the cycloketones and menthol derivatives apart from each other in two points.

The method of spatial representation (nonmetric multidimensional scaling) developed by Shepard (1962 a, b) and Kruskal (1964 a, b) can be applied to any data of relatedness, such as, for example, the present chi-square or proximity values. The only requirement is that the function relating the experimental data to interpoint distances should be monotone. In some cases in this study the monotonicity is achieved by equalizing a large number of data to the same distance. In general, the experimental data are represented by the interpoint distances, and the goodness of fit is given by the 'stress' (Kruskal 1964 a).

This method of representation is obviously a powerful tool for a study of the underlying structure of the data. A solution in two or three dimensions for a large number of odours will give chemists a clue to the important molecular parameters in the determination of odour qualities.

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The Effect of Haloperidol and Chlorpromazine on the Amine Levels of Central Monoamine Neurons

By

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Haloperidol and chlorpromazine produce central pharmacological effects similar to those seen after treatment with reserpine. Unlike the latter drug, however, they have small or no acute effects on the monoamine levels in the brain. Nevertheless, haloperidol and chlorpromazine have been found to influence the monoamine metabolism by enhancing the accumulation of basic as well as acid catecholamine (CA) metabolites, possibly as a consequence of a blockade of postsynaptic CA receptors (Carlsson and Lindqvist 1963, Andén, Roos and Werdinius 1964). After reserpine treatment the amine levels of certain CA cell groups of the lower brain stem are markedly increased in the recovery phase following an initial depletion (Dahlström and Fuxe 1964). All these findings may be explained by a compensatory increase of the activity in the CA neurons due to blockade of the neurohumoral transmission from them. Therefore it was thought to be of interest to study the effects of haloperidol and chlorpromazine on the intraneuronal monoamine levels in the brain.

Male, Sprague Dawley rats (b wt 150—200 g) have been used. Two daily doses of haloperidol (5 and 2.5 mg/kg i.p.) were administered for 1, 2, 4 or 8 days. Three daily doses of chlorpromazine (each 25 mg/kg i.p.) were given for 2, 3, 4 or 8 days. The rats were kept in a temperature of +30° C to avoid the hypothermic action of the drugs. Food and water was given by a stomach tube. The noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) levels of the brains of these rats were evaluated both histochemically (see Hillarp, Fuxe and Dahlström 1966) and biochemically.

In the brains of the rats treated with haloperidol a marked increase in fluorescence intensity was observed in many NA nerve cell bodies in the ventro-lateral reticular formation of the medulla oblongata (group A1 according to Dahlström and Fuxe 1964) at all time intervals studied (Fig. 1). The cells also appeared somewhat swollen and their processes were more distinct than usual. This was sometimes true also for the processes of the DA cell bodies of the mesencephalon. The 5-HT cell bodies were unaffected. The terminal parts of the central CA and 5-HT neurones, however, showed no certain changes in their amine levels at the various time intervals studied. The same was observed also biochemically, in the whole brain for the 2-day treatment (NA: untreated 0.38 µg/g, treated 0.46 µg/g, DA: untreated 0.31 µg, treated 0.50 µg/g, 5-HT: untreated 0.23 µg/g, treated 0.27 µg/g).



Fig 1 Effect of haloperidol on the noradrenaline level of nerve cell bodies in the ventrolateral reticular formation of the medulla oblongata (group A1 according to Dahlström and Fuxe 1964) Cross-section (a) Normal rat Weakly fluorescent cells are observed $\times 120$ (b) Rat treated with haloperidol for 2 days (see text) Swollen nerve cells with markedly increased fluorescence intensity are seen $\times 110$

After chlorpromazine treatment no increases of the amine levels were observed in the NA cell bodies in the reticular formation of the medulla oblongata. However, the DA cell bodies in the arcuate and anterior periventricular nuclei showed a slightly increased fluorescence intensity. The processes of the DA cell bodies of the mesencephalic groups also appeared more distinct than in normal rats at all time intervals studied. No changes were observed in the amine levels of the NA, DA and 5-HT nerve terminals. The same was shown also in the whole brain biochemically for the 3 day treatment (NA 0.36 $\mu\text{g/g}$, DA 0.49 $\mu\text{g/g}$, 5-HT 0.27 $\mu\text{g/g}$).

It is of interest that the CA cell bodies showing a marked increase of the amine levels after haloperidol were the same as those showing an increase after reserpine. This supports the view that blockade of the CA transmission causes, by a compensatory feed back mechanism, an increased activity in the presynaptic neuron. This may produce, besides, an enhanced synthesis of the transmitter in the terminals, also an increased formation of amine storage granules and amine synthesizing enzymes in the cell bodies.

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